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Advanced Seminar on Clinical Biochemical Pharmacology

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cancer treatment reports

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2. **Correspondence regarding manuscripts previously published in *Cancer Treatment Reports*. Letters on other topics will be published at the discretion of the Editorial Board.**
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Begin the article with a short summary.

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Submit an original and three copies of the manuscript, typed double-spaced. Use accepted spelling, abbreviation, and hyphenation found in *Webster's New International Dictionary*. Footnotes should be typed at the bottom of the page on which they appear.

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Letters published in this section can be no longer than 300 words and can be submitted with no more than 1 table and 5 references, and no figures. A second table will be permitted only when requested by the editorial board. Tables suggested for Clinical Trials Letters include one of the following: *patient characteristics and prognostic factors* (age/sex, performance status, stage of disease, pathologic types of disease [including subtypes or grade], prior therapy [number of previous regimens failed]); or *drug regimen* (dose and schedule, particularly for combination or complex regimens, number of cycles or doses/patient [mean, median, range]); or *toxicity* (standardized toxicity grades [ECOG or CALGB] or individualized grades devised and defined in the text [should demonstrate that an appropriate, biologically active dose was administered]).

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Advanced Seminar on Clinical Biochemical Pharmacology of the French-American Agreement

Sponsored and organized by the Subcommittee of Clinical Biochemical Pharmacology

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French-American Agreement Advanced Seminar on Clinical Biochemical Pharmacology

Sponsored and organized by the Subcommittee of Clinical Biochemical Pharmacology

Villejuif, France

November 29–30, 1979

Chairman: Enrico Mihich, M.D., Ph. D.

INTRODUCTORY REMARKS

The goal of the Seminar was to discuss studies of multiple pharmacological and biochemical determinants of drug action that may provide a basis for measuring these parameters in man and tailoring chemotherapy regimens in individual patients, designing regimens to exploit the modulation of antimetabolite action by metabolites, and developing new drugs and treatments stemming from concepts developed in these studies.

Although cancer chemotherapy has achieved considerable successes in the past 30 years, in sufficient selectivity against tumors by available agents and occurrence of clinical resistance are still major obstacles that need to be overcome before chemotherapy may be broadly and effectively used in the definitive management of cancer.

To reduce these difficulties, considerable efforts are being placed on the development of new drugs, drug combinations and drug combinations used with other treatment modalities. New approaches are being pursued in radiotherapy and radiosensitization, an integrated program on biological response modifiers is being activated in attempts to exploit host defense mechanisms for therapeutics, and new applications of physical and nutritional treatments are being studied. These avenues are bound to lead to improvements in the overall effectiveness of cancer therapeutics.

Among the factors contributing to an expanded role of clinical biochemical pharmacology in cancer therapeutics are the following: (*a*) advances in knowledge of the biochemical pharmacology of certain anticancer drugs in experimental systems, (*b*) the availability of increasingly powerful analytical methods, and (*c*) refinements in protocol design reflecting increased awareness by clinical cancer chemotherapists of the potential advantages of applying biochemical pharmacological concepts in designing treatment regimens.

In simplified terms, new applications of biochemical pharmacology enter clinical chemotherapy through a process that involves acquisition of information on the mechanisms of selectivity of action of drugs in experimental systems, identification and measurement of indexes of these mechanisms in humans, and rigorous verification of the validity of these indexes in cancer patients through evaluation of the therapeutic effectiveness of a particular drug in clinical protocols designed to answer specific questions related to these comparisons. The prediction of response in individual patients leading to the design of tailored regimens and the design of combination regimens based on "metabolic modulation" both follow this developmental process, and both must take into account the basic notion that in most cases multiple systemic pharmacokinetic and biotransformation factors and biochemical pharmacological factors in target normal and tumor cells are instrumental in determining selectivity of antitumor response.

The development of such biochemical pharmacological approaches and the verification of their clinical validity were discussed at the Seminar with emphasis on the progress achieved under the aegis of the USA–France Agreement and in expectation of the possibility that this area of activity may be expanded, not only through further strengthening of the Center in Marseille but also through the involvement of other French and American Centers with demonstated chemotherapeutic excellence. The proceedings of the Seminar are essentially represented by the papers reported in this issue of Cancer Treatment Reports.

E. Mihich
Buffalo, New York 14263
April 11, 1980

On the Rational Development of a New Drug: The Example of the Fluorinated Pyrimidines

Charles Heidelberger[1,*]

SUMMARY

The specific rationale for the development of the fluorinated pyrimidines and the predictions of their mode of action have been described. The mode of action of 5-FU involves incorporation into RNA and metabolic activation to 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), which inhibits the essential enzyme for DNA synthesis, thymidylate synthetase. I believe that the latter effect is responsible for the major chemotherapeutic activity. Our studies on the mechanism of the inhibition of that enzyme have led to the demonstration that a ternary covalent complex of enzyme, inhibitor, and cofactor is formed that results in essentially irreversible inhibition. The nature of this ternary complex has enabled us to devise ultrasensitive competitive ligand binding assays for thymidylate synthetase, FdUMP, and dUMP, the substrate of the enzyme. These determinants will be studied in needle biopsy specimens obtained from cancer patients undergoing chemotherapy with 5-FU in a prospective clinical investigation.

[Cancer Treat Rep 65 (Suppl 3):3–9, 1981]

The number of cases in the literature are few in which a new drug was designed on a purely rational basis, its mode of action predicted in advance, and following synthesis and testing, was developed into a clinically useful drug that exerts its action as predicted. 5-FU is one such case, and I have previously reviewed this topic (1–3).

The story started when I read a paper by Rutman et al. (4), in which they reported that uracil was used as a precursor of the nucleic acid pyrimidines of a chemically-induced rat hepatoma. This aroused my curiosity, since orotic acid was considered to be the major building block. In order to ask whether the enhanced utilization of uracil was general and/or specific for tumors, we compared the incorporation of labeled uracil and orotic acid into the nucleic acid pyrimidines of the tumor and intestinal mucosa of several transplantable tumors in rats and mice. In all cases the tumors utilized uracil to a much greater extent for DNA pyrimidines than orotic acid; in the DNA of the intestinal mucosa there was about equal utilization (5), suggesting that the enhanced uptake of uracil might be general, but not entirely specific, for tumors.

I then reasoned that it would be desirable to devise an antimetabolite that resembles uracil as closely as possible and with only one molecular modification, hoping that it would be specifically taken up by tumors, in analogy with uracil. In view of the fact that substitution of a carbon-bound hydrogen by a fluorine atom converts salad dressing into a commercial rat poison, I decided to make such a substitution in the uracil molecule. This could only occur at carbons 5 and 6, and substitution at the 5-carbon was chosen because the compound appeared easier to synthesize and for other specific reasons to be mentioned. Thus, the 5-FU molecule was selected to be the goal. The structures of uracil and 5-FU are shown in figure 1. Because the small size of the fluorine atom resembles that of hydrogen, I predicted that 5-FU should be incorporated into RNA and not into DNA in analogy to uracil. Because of the inductive effect of the strong carbon-fluorine bond, I predicted that the proton on N-3 should be more acidic than uracil, which might strengthen or weaken binding to critical sites. Finally, because it is known that DNA thymine, an essential building block, is made by the attachment of a one-carbon unit to position 5 of the uracil ring, I predicted that 5-FU should inhibit this reaction. I then synthesized a small amount of 5-FU in my laboratory at the University of Wisconsin, and managed to persuade the pharmaceutical firm of Hoffman La Roche to become interested in developing this compound as a

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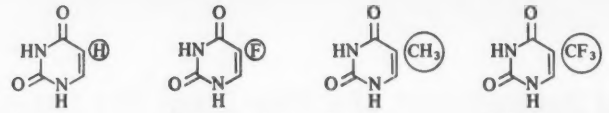

FIGURE 1.—Structures of two fluorinated pyrimidines and their normal counterparts.

potential tumor inhibitory drug. Dr. Robert Duschinsky was assigned to the project, and he improved my original synthesis by using potassium instead of sodium in two steps. This synthesis is still used in the factory today (6).

The compound turned out to be highly active against a number of rat and mouse tumors (7), and the first publication on the subject of 5-FU reported that clinical trials had started (8). The first patient was given 5-FU only one year after the first mouse: something that is no longer possible today. The initial clinical trial at Wisconsin demonstrated that 5-FU produced objective regressions of advanced breast and colon tumors (9). Subsequently this drug has been and is being used ever more frequently in clinical cancer chemotherapy. It is beyond the scope of this paper to review the clinical utility of 5-FU.

The ribonucleoside of 5-FU, 5-fluorouridine (FUR) was synthesized and found to be highly toxic and less effective against rodent tumors than 5-FU, whereas the deoxyribonucleoside, 5-fluoro-2′-deoxyuridine (FUdR) was more effective and less toxic than 5-FU (10). FUdR has also found utility in cancer chemotherapy. Later on we played the same game and substituted three fluorine atoms for the hydrogens on the methyl group of thymine to give trifluorothymine (fig 1), which was converted into the nucleoside, trifluorothymidine (F_3TdR) (11). This compound has some clinical effect in cancer and has been released as an effective drug when given locally for herpes infections of the eye (12). This compound is beyond the scope of this review, but I have reviewed its mode of action elsewhere (13,14).

We then instituted a large series of studies to verify the original predictions and determine the mechanism of action of 5-FU. On incubation of Ehrlich ascites tumor cells with 5-FU-2-^{14}C, we found no radioactivity in the DNA, and after hydrolysis and chromatography (fig 2) we found no radioactivity in the normal RNA pyrimidines, but only in a peak identified as fluorouridine-2′,3′-phosphate (15). This verified the prediction that 5-FU would be incorporated into RNA and not DNA. When we incubated Ehrlich ascites cells with labeled formate, a specific precursor of the methyl

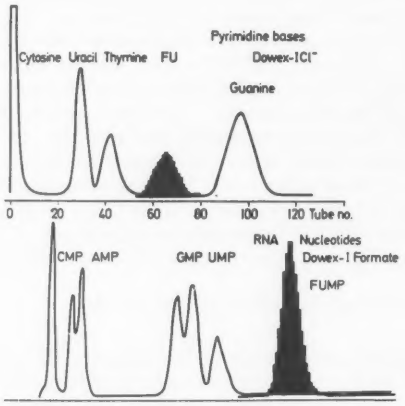

FIGURE 2.—Incorporation of 5-FU-2-^{14}C into RNA in suspensions of Ehrlich ascites tumor cells. The RNA was hydrolyzed with acid (top curve) and with alkali (lower curve), and the bases (upper curve) and nucleotides (lower curve) were separated by ion exchange chromatography. The open peaks represent A_{260} and the solid peaks represent radioactivity (15).

group of thymine, and isolated the DNA thymine chromatographically, we found that this incorporation was, in fact, strongly inhibited by addition of fluoropyrimidines to the incubation mixture (fig 3); FUdR was the most powerful inhibitor and fluoro-orotic acid was the least effective (16). Thus, the prediction that the biosynthesis of DNA thymine, and hence DNA synthesis, would be inhibited by 5-FU was verified. At this point, Dr. Seymour Cohen, who had a long experience with and had discovered the phenomenon of "thymineless death" in bacteria, was invited to investigate the effects of 5-FU. He and his group were the first to show that FdUMP was a powerful inhibitor of bacteriophage-induced thymidylate synthetase (17). In our laboratory we found that 5-FU was degraded to dihydro-FU, α-fluoro-β-ureidopropionic acid, CO_2, NH_3, and α-fluoro-β-alanine (18,19) by the same enzymes

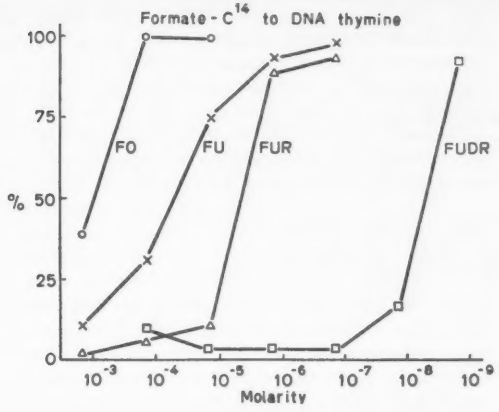

FIGURE 3.—The effect of fluorinated pyrimidines on the incorporation of formate-^{14}C into the DNA thymine of suspensions of Ehrlich ascites tumor cells (16).

that degrade uracil. We established the complete metabolism of 5-FU and F_3TdR as shown in figure 4, which was later confirmed by many others.

To summarize a great deal of work that I (3) and Mandel (20) have reviewed: the major consequences of the incorporation of 5-FU into RNA are low-probability events such as mutations in RNA viruses and phenotypic reversions in bacteriophage mutants; there are few discernible metabolic consequences of extensive substitution of 5-FU into tRNAs. Probably the result of the incorporation of 5-FU into RNA that might have most chemotherapeutic implications is an inhibition of the maturation of ribosomal RNA precursors, as discovered by Wilkinson et al (21).

Let me now consider the inhibition of thymidylate synthetase in much greater detail. The early kinetic studies of its inhibition of FdUMP showed that the inhibition was very powerful with the ratio of K_i/K_m of more than 1000 following preincubation (17,22,23). Because of this very powerful inhibition and the essentiality of thymidylate synthetase for growth, we have considered and still do believe that the main mechanism whereby the fluorinated pyrimidines inhibit tumor growth is by their inhibition of thymidylate synthetase. The reaction carried out by this enzyme is shown in figure 5. A major discovery on the mechanism of the inhibition of this enzyme by FdUMP was made simultaneously and independently in our laboratory (24) and in Santi's (25); this was the demonstration that a covalent ternary complex was formed among enzyme, inhibitor, and cofactor. This was visualized as the nucleophilic addition of a group on the enzyme to the 5, 6-double bond of FdUMP, which then attacks the methylene group of the cofactor to form the ternary complex. We found that the nucleophilic group on the enzyme is the sulfhydryl group of cysteine, and hence postulate that the structure of the ternary complex is as shown in figure 6 (26,27). If we assume that a similar ternary complex is formed with the substrate, dUMP, then a proton at the 5-position of dUMP can be eliminated along with the enzyme to regenerate the 5, 6-double bond and the enzyme sulfhydryl group. However, in the FdUMP complex there is a fluorine atom at the 5-position that cannot be so eliminated, and consquently the inhibitor complex is frozen in an irreversible situation. The mechanism of thymidylate synthetase has been reviewed by Danenberg (28).

Let us now re-examine the complexity of the metabolic conversion of 5-FU to FdUMP (fig 4). There are three pathways by which this occurs, involving a

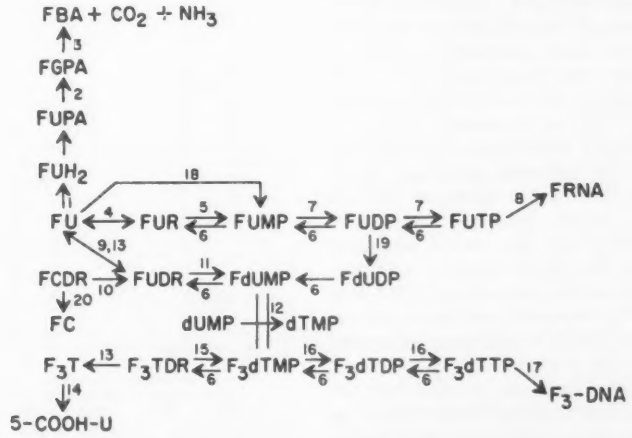

FIGURE 4.—A summary of the biochemistry and metabolism of fluorinated pyrimidines.

$$dUMP + 5,10\text{-}CH_2H_4folate \rightarrow dTMP + H_2folate$$

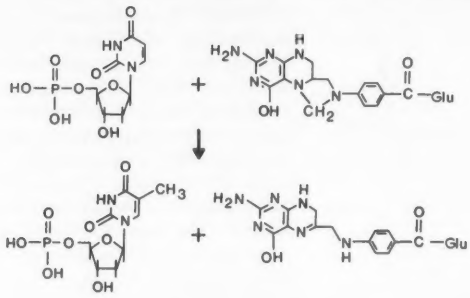

FIGURE 5.—Reaction carried out by thymidylate synthetase.

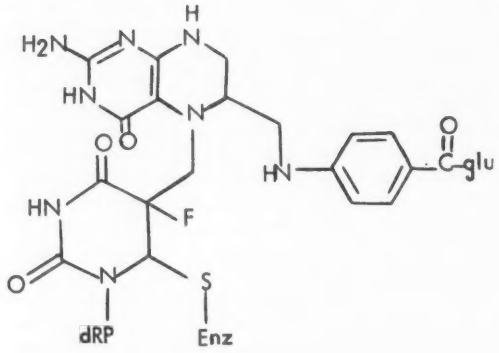

FIGURE 6.—Proposed structure of the ternary complex of FdUMP, cofactor, and thymidylate synthetase (27).

minimum of two and a maximum of five enzymes. It seems logical to assume, if the inhibition of thymidylate synthetase is the major mechanism of 5-FU's tumor inhibitory action, that the response of an individual tumor in a patient is the resultant of the quantities and activities of the various anabolic and catabolic enzymes. If this is so, then it would be highly important to determine the molecular pharmacology of 5-FU in biopsy specimens of the tumors of patients receiving 5-FU chemotherapy. If our assumptions are logical, the three parameters that should determine response should be the activity of tumor thymidylate synthetase before and during chemotherapy, the concentration of FdUMP in the tumor, and the concentrations of the substrate, dUMP, in the tumor before and during chemotherapy. The latter is important, because it has been demonstrated by Myers et al (29,30) that the concentration of dUMP in rodent tumors following 5-FU treatment builds up to such a high level that it can prevent inhibition of the enzyme.

The existence of the ternary complex of thymidylate synthetase, FdUMP, and cofactor makes possible competitive ligand binding assays sufficiently sensitive to determine accurately thymidylate synthetase, FdUMP, and dUMP in 50-mg needle biopsies of human tumors and other tissues. Dr. Richard Moran, when he was in my laboratory, developed and perfected these assays (31). The thymidylate assay involves the reaction of very high specific activity ^{3}H-FdUMP and excess cofactor with the enzyme present in a 100,000 × g supernatant fraction of the tumor, and protein-bound radioactivity in the ternary complex is separated from non-protein-bound radioactivity by suitably treated charcoal. The standard curves are shown in figure 7;

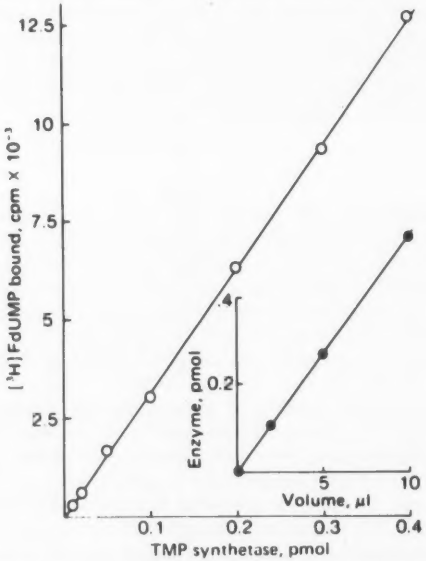

FIGURE 7.—Standard curves for thymidylate synthetase assay (31).

the assay has a sensitivity of 5 fmoles (31). The FdUMP assay involves the incubation of ^{3}H-FdUMP with pure *Lactobacillus casei* thymidylate synthetase and excess cofactor with an extract of the tumor, and the dilution of the specific activity of the labeled FdUMP by the FdUMP in the biopsy is measured, again by the charcoal technique. The standard curves are shown in figure 8; the assay has a sensitivity of 0.02 pmoles (31). The dUMP assay involves the preparation of high specific activity cofactor from ^{14}C-formaldehyde and L-tetrahydrofolate, which is purified and incubated with *L casei* thymidylate synthetase and the tissue extract. The TMP formed is separated on a minicolumn and its radioactivity determined. The standard curves are shown in figure 9; the assay has a sensitivity of 10 pmoles, which is sufficient for our purposes because of the relatively high tissue levels of dUMP (31).

The use of these methods to determine the kinetics of the response of these three parameters in cultured CCRF-CEM human lymphoblast cells to 5-FU is shown in figure 10. Following addition of 5-FU, the thymidylate synthetase activity drops rapidly, the FdUMP level increases markedly, and so does the dUMP level. These findings are similar to those reported previously in vivo (29,30).

We are now extending these studies by performing these three assays on specimens of 5-FU-sensitive and resistant mouse colon tumors and 5-FU-sensitive and resistant human colon tumor xenografts in nude mice.

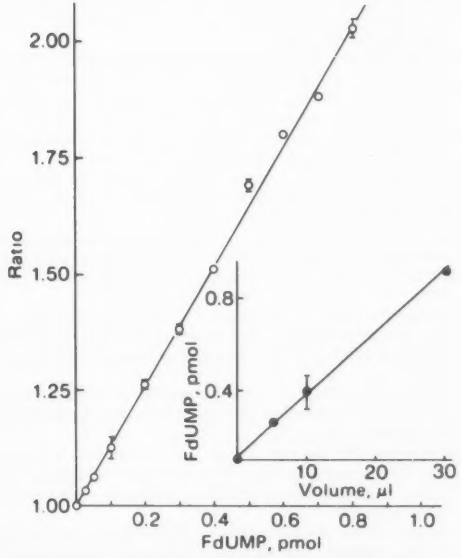

FIGURE 8.—Standard curves for FdUMP assay (31).

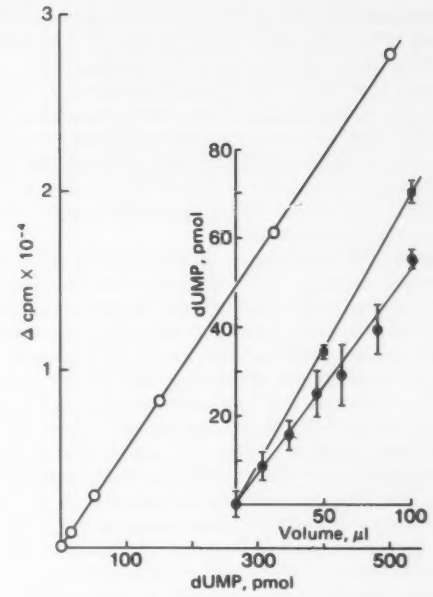

FIGURE 9.—Standard curves for dUMP assay (31).

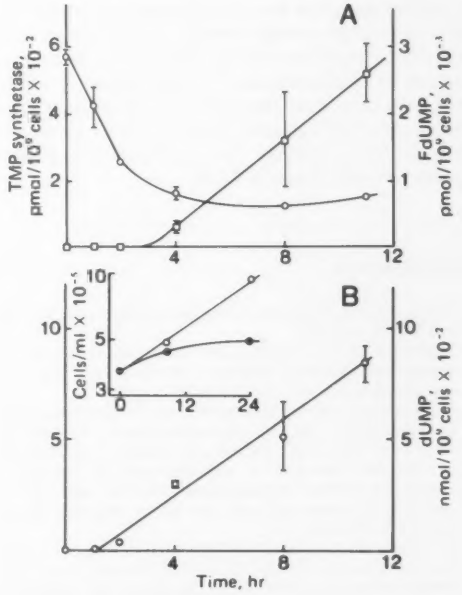

FIGURE 10.—Levels of thymidylate synthetase, dUMP, and FdUMP in CCRF-CEM cells after exposure to 30nM 5-FU (31).

Although the more micro the method the greater is the chance of sampling error, we have already determined by these ultrasensitive assays in human colon carcinomas that while there is considerable variation in thymidylate synthetase from one human tumor to another, there was not an alarming variation in activity of the enzyme sampled throughout a large and morphologically heterogeneous tumor (32). Hence, we are confident that we will obtain meaningful results from our assays of human tumors.

Armed with these assays, we plan to carry out in collaboration with Dr. M.S. Mitchell of our USC Cancer Center a prospective clinical study in patients suffering from recurrent breast and colon cancers. Needle biopsy specimens will be taken for the determination of baseline thymidylate synthetase and dUMP levels. Then the patients will be given a single chemotherapeutic dose of 5-FU, and a subsequent biopsy, or in some cases biopsies, will be analyzed for thymidylate synthetase activity and FdUMP and dUMP concentrations. The data will be collected and stored, the patients will be treated with three courses of single agent 5-FU chemotherapy, and their clinical responses will be evaluated. Such a study will provide evidence as to whether these three determinants are involved in the therapeutic response. If so, then such assays could be used to predict for the responses of individual patients to 5-FU chemotherapy. In order to make the study more comprehensive, we are in the process of developing equally sensitive assays for methylenetetrahydrofolate and other tetrahydrofolates, thymidine, and 5-FU incorporated into RNA. With such a battery of tests run on small biopsies of human tumors it will be possible to determine the molecular pharmacological mechanism of 5-FU chemotherapy in all its aspects in individual human tumors. This ability should greatly improve the clinical use of 5-FU.

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Clinical Concepts Derived from Animal Chemotherapy Studies

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SUMMARY

Animal chemotherapy studies have contributed significantly to clinical concepts in tumor therapy. Preclinical investigations have led to the discovery of new drugs and have demonstrated that it is possible to cure advanced metastatic neoplasia. A fundamental clinical concept stemming from animal chemotherapy studies is that increased selectivity and improved therapeutic effectiveness of antitumor agents may result from appropriate pharmacologic, biochemical, and biologic manipulation of the host-tumor-drug relationship. Clinically important factors that may increase antitumor drug selectivity are reviewed and pertinent studies in animal model systems are cited.

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Animal chemotherapy studies have played a vital role in advancing the treatment of clinical neoplasia. It is the objective of this report to highlight clinical concepts derived from such studies. A primary concept is that drugs that are discovered to be active in chemotherapy test systems are valid candidates for clinical trial since there is a reasonable likelihood that they will be active against clinical neoplasia. Historically, a major contribution of animal chemotherapy screening and evaluation programs has been the discovery and development of drugs that are effective in the treatment of cancer.

Another major concept derived from animal chemotherapy studies is that chemotherapy can control and cure advanced neoplastic disease. Only 20 years ago (1) it was demonstrated that treatment of advanced disseminated leukemia L1210 with halogenated derivatives of amethopterin, initiated several days prior to the death of untreated controls, was capable of extending indefinitely the life span of a significant percentage of the animals. Similarly, there are a number of advanced cancer cases in which drugs have been responsible for some patients achieving a normal life span (2,3). As emphasized by DeVita et al (4) the success of modern cancer treatment has been such that "In 1977, fully 41 percent of the 700,000 patients who developed cancer could expect to be cured." With the inclusion of patients with skin and in situ cervical cancer the "cancer cure rate increases to 58 percent."

The search for new and more effective antitumor agents therefore has a firm historical basis, and the search continues. The Division of Cancer Treatment (National Cancer Institute) has a new prospective screening program involving murine tumors growing in ordinary mice and corresponding human tumors growing in athymic mice, and screening programs are also underway in other institutions. Because of these programs, new antitumor agents are continually being discovered. It is hoped that some of them will prove highly important in the treatment of refractory solid tumors.

MANIPULATION OF THE HOST-TUMOR-DRUG RELATIONSHIP

Animal chemotherapy studies show that the antitumor effectiveness of a drug is not fixed, but may be manipulated. Increased selectivity and improved therapeutic effectiveness can be achieved by taking into account the complex interrelationships of the host, tumor, and drug (5–8). There are pharmacologic, biochemical, and biologic factors that may influence the antitumor selectivity of drug action and the net therapeutic effect. Some of the major factors are outlined below.

Dosage, Schedule, and Route

The principle has emerged that all cancer chemotherapeutic agents selected for clinical trial should be investigated in experimental systems with respect to the effect of dosage, scheduling, and route of administration.

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Drug Dosage

The antitumor effectiveness of a drug is governed by the dose-response relationships for both the host and the tumor. For most of the available antitumor agents the dose-response curves are steep and rise quickly to lethal toxicity, thereby limiting the total dosage that can be employed. Characteristically, as the dose of an active drug such as methotrexate is increased, the survival time of the animals increases relatively steeply until a maximum effect is achieved at an optimal dose. When the optimal dose is exceeded, the survival time diminishes, also usually relatively steeply, as the result of toxicity of the drug. This type of study provides information for clinical consideration pertaining to the optimal dosage, the relationship of dose to response, and the relationship of dose to host toxicity (5,9–11).

It is well established that studies of dose-response relationships in experimental models may predict clinical risks for both quantitative toxicity, including safe starting dose, and qualitative toxicity. The experimental models employed to evaluate the toxicity of potential anticancer drugs provide results that correlate closely with the results in man. Freireich et al (12), in a comparison of the quantitative toxicity of 18 anticancer agents in the mouse, rat, hamster, dog, monkey, and man, determined that the maximum tolerated dose per square meter of body surface area (mg/M^2) was the same in each of the animal species and in man.

Preclinical observations of qualitative toxicity provide the clinical investigator, at a minimum, an indication of the toxic manifestations that may occur and whether they are predictable, reversible, and manageable (13–17).

Scheduling Characteristics

An important clinical concept from animal chemotherapeutic studies is that the schedule of administration may effect markedly the therapeutic outcome (18,19). Determining the schedule dependency of drugs may provide a basis and new leads for improved therapeutic effectiveness. Such studies may encompass the determination of (*a*) optimal number of treatments; (*b*) optimal interval between treatments; (*c*) optimal total duration of treatment, and (*d*) optimal interval between courses of treatment if the treatment is interrupted. Optimal scheduling of drug administration involves pharmacologic and cytokinetic considerations encompassing drug specificity for tumor and host and differential host recovery.

The possibility of a close correspondence of animal and clinical activity is illustrated by the observation that intermittent high dose treatment with methotrexate is considerably more effective than daily treatment both in increasing the survival time of mice with leukemia L1210 and in prolonging remission and life span of children with acute leukemia (20–22).

Leukemia L1210 studies also demonstrated the high effectiveness of cytosine arabinoside when administered on intermittent schedules (23,24). Intermittent therapy also appeared to improve the therapeutic effectiveness of cytosine arabinoside against acute myelocytic leukemia (25,26).

Pharmacokinetic characteristics of drugs and biochemical and cytokinetic mechanisms of action may be important determinants of schedule dependency (27–30). Skipper et al (27) predicted the schedule dependency of the ribonucleoside diphosphate reductase inhibitors guanazole, hydroxyurea, and 5-hydroxy-picolinaldehyde-thiosemicarbazone by correlating data on biochemical sites of action, kinetics of tumor cell proliferation, maximum achievable levels in body fluids, minimum levels necessary for effective tumor cell destruction, and the effect of drug concentration and time at the target cell on the rate of tumor cell kill.

The pharmacokinetic characteristics of drugs may have an important influence on optimal scheduling. A slow release form of cytosine arabinoside such as cyclocytidine may not share the schedule dependency characteristics of cytosine arabinoside (28,29). The time required for host recovery from the toxic effects of a chemotherapeutic agent relative to the tumor cell population may also be an important factor in determining optimal scheduling.

Route of Administration

Preclinical investigation has shown that the route of administration may influence the therapeutic effectiveness of drugs. In correspondence with the clinical observation, dichloromethotrexate, which is markedly effective in the treatment of advanced leukemia L1210 on parenteral administration, dropped sharply in therapeutic effectiveness when administered orally (31).

The Body Burden of Tumor Cells

A clinically important contribution from chemotherapy studies is that the total body burden of tumor cells may effect the therapeutic outcome profoundly.

The relationship between size of leukemic (L1210 and L4946) inoculum and chemotherapeutic cures with amethopterin has been demonstrated definitively (32–34). The higher the leukemic challenge the greater was the dosage of amethopterin required to eradicate the leukemic cell populaton.

Goldin et al (35) suggested that the eventual death of leukemic mice could in some instances be due to the slow but persistent growth of sensistive tumor cells (background growth) in the face of treatment. This concept of background growth of sensitive cells in a drug-treated animal was discussed in more detail by Klein

(36). Friedkin and Goldin (37) developed mathematical models to relate the interplay of factors such as life span of leukemic animals, backgrond growth, and drug resistance (including fractional survival and prolongation of generation time, time of initiation of treatment, survival of sensitive and resistant leukemic cells in treated animals, incidence of mutation from the sensitive to the resistant state, and dihydrofolate reductase levels in mixed populations of sensitive and resistant cells).

Skipper and colleagues (38) conducted extensive studies on the criteria and kinetics associated with the curability of experimental leukemia, of general pertinence to therapy of solid tumors. The following generalizations emerged from these studies:

One leukemic cell is lethal.—Since cure is the goal, a most important guideline or value regarding the response of leukemia to therapy is the number of leukemic cells that survive treatment.

The dynamics of the proliferation of L1210 leukemic cells involves an approximate 2-day lag phase associated with transplantation.—This is followed by log-phase proliferation until host death, at which time the host's leukemic population is of the order of 1 billion cells. Leukemic cells eventually enter the brain when the host's peripheral leukemic population is about 10 million cells.

The biologic events associated with marked increase in life span of treated leukemic animals may be viewed as follows.—(*a*) Single dosage therapy: a drug kill of more than 99% of the leukemic cells and a delay in host death approximately proportional to the time required for the surviving cells to proliferate to a lethal number. (*b*) Daily treatment until death from the disease: interplay between daily drug kill of some percentage of the host's leukemic cells and proliferation of the "escapee" cells, with the latter exceeding the former.

The blood-brain barrier.—This can play an important role in drug failure, as can heterogeneity of the individual leukemic cells with regard to drug sensitivity during periods of maximal blood and tissue drug levels.

Fractional cell kill hypothesis.—The percent of varying sized leukemic cell populations killed by a specific dose of an effective drug is reasonably constant (fractional cell kill hypothesis) if the cell population is metabolically homogeneous and all cells are exposed to the same drug concentration for the same amount of time. A maximum single dose of an agent that will reduce a 10^4 cell population by 4 logs to an average of one leukemic cell per animal (99.99% cell kill) may result in 40% of a large group of animals having zero surviving leukemic cells (Poisson distribution law). On the other hand, the same dose administered to animals bearing 10^6 leukemic cells might result in an average of 10^2 surviving cells per animal and no animals with zero cells.

Dosage.—There is a close relationship between dose level and the percent of a leukemic cell population killed by an effective drug. Since the toxicity of most antileukemic agents to experimental animals is cumulative and since the proliferation of the surviving leukemic cells rapidly replaces those killed by relatively low chronic doses, it appears that high-level, short-term schedules offer considerably greater potential for obtaining "cures."

Not only with increasing inoculum but also as the disease becomes more advanced, there is generally a loss in therapeutic effectiveness in increasing both survival time and cure rate. This was observed for drugs such as guanazole, 5-azacytidine, 5-hydroxypicolinaldehyde-thiosemicarbazone (5-HP), iphosphamide, and cytosine arabinoside in treatment of advanced leukemia L1210 (39–41).

With Ridgway osteogenic sarcoma, clinical staging influenced the extent of response to therapy (42). With actinomycin D, delay in administration until the disease was more advanced resulted in a marked decrease in the percentage of complete regressions and cures. With adriamycin, delay resulted in a marked reduction in the number of partial and complete regressions.

With Lewis lung carcinoma, delay in treatment with cyclophosphamide also resulted in a marked reduction in the percentage of long-term survivors, attributable to an increase in the size of the local tumor mass at the site of intramuscular inoculation and to metastatic spread of tumor to the lung (40).

The Origin and Treatment of Tumor Cell Resistance

Experimental chemotherapeutic investigations have provided concepts pertaining to the origin of tumor cell resistance, its prevention, and the potential for effective treatment.

An important obstacle to successful therapy, particularly pertinent where the disease is advanced and therapy is protracted, is the development of tumor cell resistance. The selective toxicity of antitumor agents and the extent of tumor cell resistance are governed in general by the same pharmacologic, biochemical, and biologic factors but act in either a favorable or unfavorable direction. The rapidity with which a resistant tumor cell population originates may be influenced by the inherent tendency to spontaneous mutation, the initial degree of sensitivity of the tumor cells, the number of tumor cells in the population, and the mutagenicity of the drug employed, as well as its chemotherapeutic effectiveness.

The presence of metastatic disease at the initiation of therapy, as well as metastatic disease during therapy, may result in the dual obstacle of increase in tumor cell burden and sequestration of metastatic foci. DeVita et al (3) have emphasized that when cancer is initially de-

tected as a 1 cm^3 mass, a billion cancer cells may already be present. At this somewhat advanced stage of development of the disease, both a population of spontaneous mutants to various drugs and metastases are undoubtedly present.

Animal studies have indicated that a small number of resistant cells in a tumor cell population may result in therapeutic resistance. In one study with leukemia L1210 there was a marked drop in therapeutic response to methotrexate in a mixutre of 1 resistant cell per 1000 sensitive cells (43).

Several reviews on the mechanisms of resistance to antitumor agents (44–54) indicate the value of pharmacologic and biochemical studies in contributing fundamental information of clinical interest and conceptual value. In the report of Mihich et al (52) of an International Union Against Cancer workshop, it was emphasized that appropriate evaluation of metabolic and pharmacodynamic determinants of drug action in target cells may provide a basis for predicting sensitivity or resistance of a human tumor to specific drugs and selecting optimal dose regimens or combination therapies for individual patients. Major areas of interest for potential improvement of drug specificity and diminuation of resistance delineated at the UICC workshop include uptake of drugs and precursors, enzyme activity and nucleotide pools, DNA repair and drug-induced toxicity, and rational approaches to clinical chemotherapy.

Skipper and associates (53,54) advanced the following treatment concept for controlling drug-resistant tumor cells. With a number of experimental tumors, treatment of advanced disease initially results in tumor regression, but tumor growth resumes despite continued treatment. This has been observed, for example, with actinomycin D, cis-dichlorodiammine platinum, and 5-FU in the treatment of advanced Ridgway osteosarcoma and with the combination of adriamycin and 5-FU in the treatment of advanced mammary adenocarcinoma line 16/c. The regrowth phenomenon has been observed with single drugs and combinations of drugs representing the various chemical and functional classes of antitumor agents. Usually the resumption of growth during chronic treatment with a drug or drug combination is attributable to the overgrowth of tumor cells that are resistant to the single drug or to one or more of the combined drugs. The occurrence of regrowth of a resistant tumor cell population is dose-dependent. In one experiment, treatment with an LD_{10} dose of cyclophosphamide plus 6-mercaptopurine cured all of the mice bearing Ridgway osteogenic sarcoma, whereas with one third of the curative doses of this drug combination the initial regression of all tumors was followed by resumed growth despite continued therapy (55).

Schabel et al (54) consider that adding more anticancer drugs in combination chemotherapy may not resolve the problem of overgrowth of drug-resistant tumor cells, since resistance to multiple drug combinations has been observed, individual drug doses may have to be reduced as additional drugs are added to drug combinations, and the development of resistance to one or more drugs in a combination may result in undesirable drug toxicity on continued treatment with these drugs.

Skipper and associates (53,54) have concluded that a logical conceptual approach is to eradicate a maximum number of drug-sensitive cells with a limited number of effective though noncurative drugs, then change to other drugs that are cytotoxic both to residual tumor cells sensitive to the first treatment as well as to mutant tumor cells that have become resistant to the first treatment and were growing despite continuous thera py. Second-generation therapy should be initiated about the time when the numbers of drug-sensitive and drug-resistant tumor cells are equal. This approach may be more therapeutically effective than protocols in which therapy is terminated at the time of apparent disappearance of tumor and is not reinitiated until tumor regrowth is evident.

This sequential therapy approach has been supported by a number of studies in model tumor systems. In one study 10^7 L1210 leukemic cells were inoculated intravenously. On treatment with cytosine arabinoside plus 6-thioguanine therapeutic synergism was obtained, but treatment ultimately failed since the animals died with tumor cells resistant to both drugs. However, two courses of cytosine arabinoside plus 6-thioguanine therapy followed by cyclophosphamide plus BCNU produced marked increases in life span and a high percentage of long-term cures (53). Similar results have been obtained with sequential treatment in a number of studies with leukemia P388 (54).

It is important in this type of sequential therapy that the drugs not show cross-resistance. Information concerning cross-resistance characteristics is generally available from animal studies (54,56). Also, drugs chosen for sequential therapy could be those that have evidenced collateral sensitivity in animal tumor models. There is by now considerable information on drugs that yield collateral sensitivity following the origin of resistance to initial therapy (54,57).

Thus experimental investigations are contributing concepts for the control and therapy of drug-resistant tumor cells, with respect to choice of drugs, scheduling of therapy, and in overall planning of clinical chemotherapy protocols.

Combination Chemotherapy

Both preclinical and clinical studies have demonstrated clearly that combination chemotherapy

with two or more drugs may be more therapeutically effective than drugs employed individually (therapeutic synergism). The biological basis for increased antitumor specificity with combination chemotherapy may be summarized as follows (58,59).

Increase in Antitumor Effectiveness Without a Proportionate Increase in Limiting Toxicity for the Host

The drugs may be selected by these criteria: (*a*) they are effective individually; (*b*) they exert differing qualitative toxicities or pharmacologic activities; (*c*) they act by different biochemical mechanisms, which may result in favorable supplementary action against the tumor.

Schabel (60) has observed that for a series of chemotherapeutically synergistic drug combinations, the host toxicity of the drugs was usually less than additive. An example in which a combination of drugs exerted therapeutic synergism involves methotrexate plus 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in the treatment of systemic leukemia L1210 (61). With this combination, too, the toxicity of the drugs was less than additive, permitting the use of a higher total dosage.

Proposed mechanisms of multiple biochemical blockade and cooperative biochemical interactions between two active drugs that may lead to improved antitumor selectivity have been summarized as follows by Mihich and Grindey (62).

Sequential blockade: inhibition of two sites along a linear sequence in a series of biochemical transformations (63).

Concurrent inhibition: Inhibition of two sites on alternate pathways involved in the synthesis of the same product or macromolecule (64).

Complementary inhibition: one drug inhibits the biosynthetic pathway to a macromolecular target (DNA, RNA, protein) and the other directly damages the macromolecular polymeric target (65-67).

Single enzyme inhibition: binding of the first drug to the enzyme increases the binding affinity of the others (68,69).

Concerted inhibition: inhibition of two sites within a regulated metabolic framework (69).

Enhanced activation: the effects of one drug favor the activation of the other drug (70-72).

Decreased inactivation: one drug inhibits the metabolic degradation of the other (73).

Increased uptake: one drug may increase the cellular or organelle uptake of the other. Vincristine was observed to augment the steady state for methotrexate in L1210 tumor cells in vitro (74, 75) although it did not alter the initial rate of methotrexate uptake (75).

Decrease in Host Toxicity Without a Proportionate Loss in Antitumor Effectiveness

Examples include methotrexate plus delayed administration of citrovorum factor (35), cytosine arbinoside plus 6-thioguanine (60), ICRF-159 plus 6-thioguanine (76), N-acetyl cysteine plus iphosphamide (77).

Identification of Optimal Dosages and Dosage Ratios

Optimal therapy with optimal dosage of methotrexate plus markedly reduced levels of 5-FU (78).

Determination of Optimal Scheduling

In treating leukemia L1210, 6-mercaptopurine plus azaserine administered every 2 days was superior to daily treatment (79), and simultaneous treatment with cytosine arabinoside plus 6-thioguanine was superior to sequential drug treatment (60).

Delay in Origin of Resistant Mutants and Treatment of Tumor Cell Resistance

Cytosine arabinoside plus cyclophosphamide delayed the appearance of resistance to cytosine arabinoside (80).

Treatment of Metastatic Disease Including Sequestered Tumor Cells

BCNU and cytosine arabinoside are therapeutically active against both subcutaneous and intracranial leukemia (81), which may provide a basis for therapeutic synergism where there is intracranial disease. Cyclophosphamide plus methyl-CCNU elicited therapeutic synergism against advanced Lewis lung carcinoma (82).

Kinetic Considerations

Tumor cell synchronization: vinblastine and also colcemid synchronized L1210 cells and therapeutic synergism was observed when cytosine arabinoside was administered during S-phase (83).

Drugs that act at different phases of cell cycle: cell-cycle-stage-specific action of Ara C plus non-cell-cycle-stage-specific action of nitrosoureas (84).

Drugs to stimulate G_0 cells to divide: to increase tumor cell susceptibility to a second drug.

Loading dose chemotherapy of advanced disseminated tumor: To reduce body burden of tumor cells and increase proportion of actively dividing cells (85,86).

Drugs that assist active compound: Prevention of detoxification, maintainance of blood and tissue levels, improved penetration to target tumor site, or other means for increasing C×T.

Search for drugs that may act against the carcinogenic activity of antitumor agents, against tumorogenic virus or virus induced tumors, plus antitumor drugs.

Polychemotherapy: Despite the strong clinical interest in polychemotherapy there have been few studies of polychemotherapy in preclinical systems. A model has been reported for combination chemotherapy with three drugs (87), but the methodology has not been exploited.

Adjuvant Chemotherapy

The preclinical and clinical evidence to date has indicated clearly that the combined modality of surgery plus chemotherapy (adjuvant chemotherapy) with individual drugs and with drug combinations, by reduction of tumor cell mass provides a means for circumventing and overcoming the obstacles to totally eradicating the tumor cell population in the host (88-106). Instances of preclinical investigations involving adjuvant chemotherapy may be cited.

Martin and his colleagues (105,106) employing the $CD8F_1$ murine breast cancer model showed that tumor recurrence could be prevented following surgery by using chemotherapy or the crude immunostimulant zymosan.

Surgery plus treatment with 6-mercaptopurine was more effective than surgery alone or 6-mercaptopurine alone in curing animals with adenocarcinoma 755 (88). With surgery followed by treatment with methyl-CCNU, there was an incidence of 50% survival in the treatment of B16 melanoma, while with surgery alone or methyl-CCNU alone none of the animals survived (89). Surgery plus treatment with cyclophosphamide was more effective than surgery alone or cyclophosphamide alone in the treatment of mice carrying Lewis lung carcinoma (90-92). Surgery plus cyclophosphamide plus methyl-CCNU was more effective than surgery alone or treatment with drugs individually or in combination in curing animals with advanced Lewis lung carcinoma (90). The combination of surgery plus the four-drug combination 6-thioguanine, cyclophosphamide, actinomycin D, and mitomycin C (TEAM) was more effective than surgery alone in yielding local cures and absolute cures in animals bearing spontaneous breast tumors in autochthonous hosts (93). Fugmann et al (94) found that phenylalanine mustard plus 5-FU decreased the incidence of mice with lung metastases.

After surgery there may be an increase in the proliferative fraction of cells, which may result in enhanced drug sensitivity (95).

Thus the generalization for the clinic is clear, that surgically reducing tumor burden may permit chemotherapy to be more effective. Conversely, prior chemotherapy, where appropriate, may be sufficiently effective in treating disseminated disease to permit successful surgical extirpation of remaining localized tumor. The preclinical investigations may provide recommendations for the clinic concerning drugs that may be utilized in surgical adjuvant chemotherapy.

It is important to conduct studies on dosage and scheduling of drugs in relation to surgery. Skipper (98) has shown that solid tumors enlarge in accordance with Gompertzian growth kinetics. Both Norton and Simon (96) and DeVita (97), in extending the studies of Skipper and colleagues on the criteria associated with curability, suggested that since most human tumors grow in Gompertzian fashion both very large and very small tumors may be less sensitive to treatment with effective drugs than tumors of intermediate size. A greater absolute number of tumor cells might be killed by drug treatment at the intermediary inflection point than when the tumors are larger or smaller, since the Gompertzian function states that the rate of growth (increase, in mass per unit of time) is lowest for both the very large and very small tumors. This interesting concept is worth investigating. Regarding adjuvant chemotherapy, where the tumor cell population has been reduced markedly so that essentially only metastatic foci of disease remain, it is considered that intensive schedules involving high dosages as well as more prolonged therapy than usual would provide regimens that could be most effective in obtaining cures. Data from extensive trials with metastatic solid tumors of mice have clearly demonstrated the importance of high-dose adjuvant chemotherapy to obtain maximum cures after surgical removal of the large primary tumor (103).

Preclinical experience indicates that a number of advantages may result from the utilization of chemotherapeutic adjuvant treatment (99). Where metastatic disease is not too extensive, reduction of the body burden of tumor cells by surgery may increase lifespan sufficiently to permit effective drug treatment. Surgical removal of a tumor may improve host tolerance to a drug so that higher doses may be used, reduce any immunosuppressive effect of drug treatment, retard metabolic depletion of the host, and diminish immunosuppressant action on the host resulting from progressive tumor growth. It may also reduce the incidence (absolute number) of spontaneous and drug induced mutants and reduce the extent of metastatic spread and tumor cell sequestration. Finally, by decreasing the challenge to therapy, surgery may increase drug effectiveness against tumor both in regard to effective control of tumor growth and in the possibility for total tumor cell eradication.

Radiation Plus Chemotherapy

There have been no extensive preclinical investigations with radiation plus chemotherapy, and the full

potential of this combined modality awaits determination. Yet, an early study may be cited in which whole body irradiation plus chemotherapy was more effective than either modality alone in increasing the survival time of mice with leukemia L1210 (107). The current interest in testing specific types of drugs as radiopotentiators in preclinical test systems is already contributing significantly to clinical utilization of this combined modality.

Biological Response Modifiers

Biological response modifiers alone and coupled with chemotherapy will undoubtedly have an important impact on clinical therapy.

CONCLUSION

The interest in multifaceted manipulations of the host-tumor relationship to improve the therapy of neoplastic disease stems from a broad base of preclinical experimental data. Developments in this area, with the additional stimulus provided by the new Biological Response Modifier Program in the Division of Cancer Treatment, NCI, are awaited with great interest.

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Biochemical Pharmacology in Medical Oncology

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The major science underlying cancer chemotherapy is pharmacology. This includes basic pharmacologic efforts, such as those involving drug development and those involving studies of the mechanism of action and resistance at a cellular, biochemical, and genetic level. What I wish to present relates to clinical pharmacology. The importance of clinical pharmacology hardly requires emphasis. What I propose to do is to take three areas where there is therapeutic movement or the potential for therapeutic movement and indicate the central role that clinical pharmacology plays or should play in such research.

Regional approaches to cancer chemotherapy have been popular for a number of years, but the application of sound pharmacologic principles and study to this approach is of relatively recent vintage. For example, the fluoropyrimidines have been employed in intrahepatic artery infusion for patients with metastatic cancer, usually colorectal cancer, of the liver. The tumor regression rate was approximately 50%, which suggested that this approach was effective, since systemic administration of the same agents provides a 15%–20% response rate.

Several years ago we initiated studies integrating pharmacology and nuclear medicine into the hepatic artery infusion approach. Fundamental to this approach is the assumption of a steep dose response curve for the fluoropyrimidines. As is apparent in table 1, and in essentially all studies of the fluoropyrimidines, experimental and clinical, the dose response curve for transplanted tumors, including colon cancer, in the mouse is steep (1)[3]. Thus the regional approach, which offers the opportunity of delivering high concentrations to the tumor and relatively low concentrations systemically, is attractive. While it is commonly assumed, particularly by clinicians, that infusion of a drug into the afferent artery of a tumor will supply a higher concentration to the tumor than systematically, this is often not the case. Factors that favor drug extraction in the first capillary pass include a short plasma half-time, little or no protein binding, and lipid solubility (2). The liver has a major capacity to biotransform and/or excrete many drugs. Thus regional infusion of the liver provides a major opportunity for delivering a higher concentration of drug to the tumor, as compared to the systemic circulation. Our pharmacologic model for the study of this phenomenon in man is presented in figure 1. In this protocol, drug can be administered by continuous infusion either into the hepatic artery or to the peripheral vein. The hepatic vein can be catheterized during the pharmacologic studies. Knowledge as to the hepatic artery and hepatic vein concentrations, the amount of drug delivered, and the hepatic flow rate allows for a definitive analysis of the extraction fraction. Such analyses, particularly for FUDR and 5-FU, are made in tables 2 and 3 (3).

In this study FUDR was given by continuous administration into a peripheral vein (table 2). The blood flow across the liver was measured, simultaneous drug levels in the hepatic artery and hepatic vein were obtained, and the extraction ratio was determined. Note that regardless of dose rate, the hepatic extraction was 80%, and fairly uniform between patients. Since established metastatic tumors in the liver are largely supplied by the hepatic artery, it is assumed that tumors are exposed to the same concentration that exists in the hepatic artery. This, plus the extraction ratio, implies that during hepatic artery infusions, five times the concentration of drug is delivered to the tumor, in comparison to the hepatic vein. Based on

TABLE 1.—Colon cancer no. 38*

5-FU ip days 5–10 (mg/kg)	Tumor growth (T/C)†
0	100%
13	106
19	65
28	16
40	0

*T. Corbett, Southern Research Institute, Montgomery, AL.
†Size of tumor in treated (T) and control (C) animals measured at day 27 (T/C × 100 = 100% = no effect).

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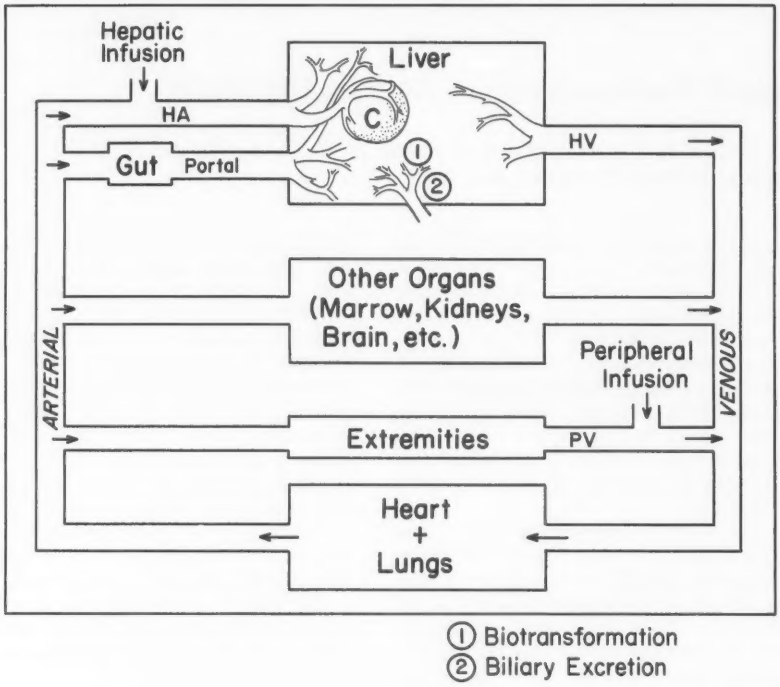

FIGURE 1.—Hepatic artery infusion kinetics. HA/HV = extraction ratio.

TABLE 2.—Hepatic extraction of FdUrd during peripheral intravenous administration

Patient	Dose*	EHBF† (liters/min)	Levels‡(μM)		Extraction ratio§	Hepatic clearance (liters/min)	Intrinsic clearance¶ (liters/min)
			HA	HV			
1	5	1.15	0.13	0.03	0.77	0.88	4.0
	20	1.15	4.2	1.2	0.71	0.81	2.8
2	0.5	2.50	0.38	0.03	0.92	2.3	29.0
	5	2.50	2.6	0.20	0.92	2.3	29.0
3	5	1.22	0.7	0.03	0.81	0.99	5.2
	20	1.22	4.0	0.45	0.89	1.1	9.9
4	5	1.69	0.52	0.15	0.71	1.2	4.1
	20	1.69	2.1	0.54	0.74	1.2	4.8
5	5	2.15	0.48	0.15	0.69	1.5	4.8

*The dose is mg of FdUrd/kg of body weight/hr via constant infusion.
†Estimated hepatic blood flow (5).
‡By radioimmunoassay of hepatic arterial (HA) and HV serum samples obtained at steady state.
§(Hepatic arterial level − HV level)/hepatic arterial level.
¶Intrinsic clearance = (estimated hepatic blood flow × extraction ratio)/(1−extraction ratio).

TABLE 3.—Hepatic artery infusion of FUDR

Estimate of tumor/peripheral blood concentration gradient

1) 20 mg/kg/hr peripheral iv
   Hepatic artery concentrations of 2-4 μM (table 2).

2) Hepatic blood flow = 0.75–1.0 liter/min (2)
   Hepatic artery supplies 1/3 (2/3 from portal vein), therefore 0.25–0.33 liter/min or 15–20 l/hr.

3) The 5.7×10^{-3} moles/hr FUDR infused into hepatic artery are diluted into 15–20 liter/hr (from 2 above); the hepatic arterial concentration with direct infusion is 3×10^{-4}M.

4) With HA infusion 3×10^{-4}M (from 3)
   With PV infusion $2\text{–}4 \times 10^{-6}$M (from 1)
   Therefore, HA infusion results in 75–150-fold greater concentration within tumor than same dose rate by peripheral vein.

5) If one titrates dose to achieve same systemic concentration (see table 6 in ref 2), one gives a fourfold higher dose rate with hepatic artery infusion.

6) Therefore $4 \times 75\text{–}150 \cong$ 300–600-fold advantage.

calculations from pharmacologic studies (2), a 300–600-fold greater concentration of FUDR is achieved in the tumor, as compared to the peripheral blood (table 3). Pharmacologic studies of 5 agents are summarized in table 4. The extraction ratio for FUDR is 80%; that is, the concentration in the hepatic artery over the hepatic vein at equilibrium is 4:1. 5-FU is substantially less effective in this setting, where there is an extraction ratio of approximately 30%, as is adriamycin, with an extraction ratio of 44% (4). Consistent with this observation is the fact that four to five times as much FUDR can be delivered into the hepatic artery as can be delivered into the peripheral vein for an equal amount of toxicity. This ratio for 5-FU is 1.5:1; for methotrexate it is 1:1; but for dichloromethotrexate, which is largely metabolized in the liver, it is approximately 1.6:1. These studies would indicate that the concentration at equilibrium of methotrexate delivered to the liver and to liver tumors is no greater when given by hepatic artery than by peripheral vein. 5-FU and adriamycin are intermediate by hepatic artery infusion (table 4). For compounds such as FUDR, the very high extraction ratio provides a highly favorable tumor/peripheral blood ratio. In view of the very steep dose-response curve mentioned above, this might be expected to result in a greater therapeutic effect.

The above is consistent with the observation that FUDR given by hepatic artery infusion produces a 50% response rate, as compared to a 15% response rate with 5-FU or FUDR when given by peripheral vein to patients with colorectal cancer (5). In one comparative study of intravenous and intrahepatic artery infusion of 5-FU, there was only a slight difference in response rate (35% as compared to 25%), but 5-FU on the basis of the above pharmacological studies would not be expected to be the optimal drug for hepatic artery infusion (6).

Another pharmacologic question we asked with respect to the hepatic artery infusion program had to do with drug flow rates. A basic assumption of the program is that the drug when infused into the hepatic artery does indeed get to the tumor and diffuses relatively uniformly throughout the tumor. This position was supported by the observation that "bolus constrast" angiography (high flow rate, short term) was generally associated with good filling of the tumor bed. Since the chemotherapeutic agent is given at a much slower rate, we examined the relative distribution of vascularity with fast and slow rate angiography, comparing contrast angiography with radionuclide angiography (technetium-99m/sulfur colloid, or technetium-99/m/stannous phytate). It was found that flow rates appropriate for FUDR delivery into the hepatic artery (generally slow) often perfused the tumor substantially less well than high-flow-rate angiography (figure 2). When such studies were performed prior to FUDR chemotherapy in 23 patients, it was found that 10 of 11

TABLE 4.—Extraction ratios of drugs given by various routes

Drug	Drug given by peripheral vein Concentration ratio,* HA:HV	Drug given by hepatic artery Concentration ratio† HA:PeV	Systemic blood levels Drug given by PeV or HA‡ PeV:HA
5-FU	1.3:1	—	1.5:1
FUDR	4:1	300-600:1§	5:1
Adriamycin	3:1	—	—
MTX	—	—	1:1
DCM	—	—	1.6:1

*Ratios not affected by dose rate over 5X range.
†Clearance and extraction values calculated from hepatic blood flow determinations (indocyanin method).
‡Or ratio of equitoxic doses.
§See table 3.
PeV = peripheral vein; HA = hepatic artery; HV = hepatic vein.

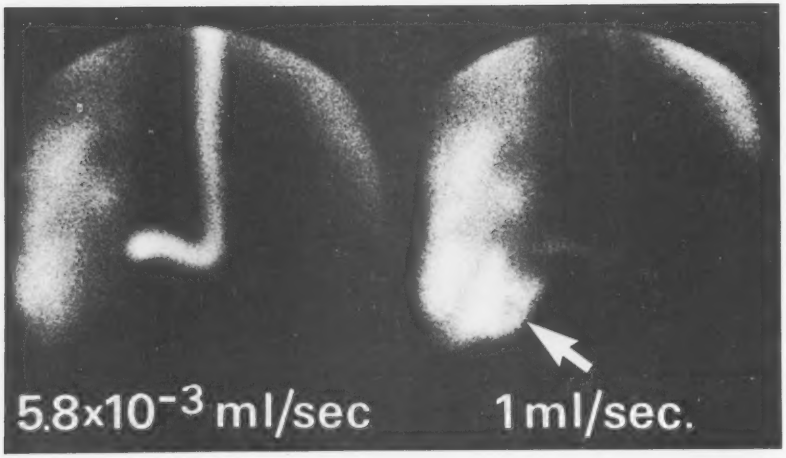

FIGURE 2.—Effect of drug infusion rate on tissue perfusion. High Infusion rates of ^{99m}Tc-MAA (right) allow radioaggregate flow to region of liver (arrow) not perfused at slower drug infusion rates (left).

with good distribution into the tumor responded (objective tumor regression), whereas 0 of 8 with poor distribution responded ($P = 0.05$). We subsequently have treated 12 additional patients with good perfusion at the flow rate employed for chemotherapy, and 11 have responded.

Thus, in circumstances where angiography predicts good perfusion of the tumor at the slow flow rate employed for FUDR, 21 of 23 (90%) have responded (8). Therefore, we have defined a major discriminant for response to hepatic artery infusion. While it remains to be determined, it seems probable that this will apply to most other drugs in addition to FUDR.

Our protocol for heaptic artery infusion has been to deliver one or two courses of treatment. If the patient responds, no further treatment is given until relapse occurs. However, continuing treatment to maximize the magnitude and duration of response is strongly indicated. The problem with this in the past has been that it has been necessary to use external pumps and a catheter that passes the skin barrier. More recently the implantable Infusaid (Metal Bellows Corporation, Sharon, MA) pump has been developed (see below). Our protocol now calls for the implantation of this pump in the subcutaneous space in the abdomen, with insertion of the catheter into the hepatic artery in patients who have responded after one or two courses of intrahepatic artery FUDR. In such patients, continuous FUDR can be delivered, and following failure, other drugs may be employed, using the same technique and route of administration. A more detailed discussion of this technique follows.

In summary, these pharmacological studies have placed on a scientific basis the hepatic artery and regional chemotherapy approach, and we propose that for new drugs such as mitomycin, platinum, AD 32, and others, that pharmacological studies be performed in parallel with the regional approach. A compound that might be particularly suitable to this approach is AD 32, since it is not protein-bound, is very extensively metabolized by the liver, is lipid-soluble, and to date has not produced cardiac toxicity with long-term administration.

CONTINUOUS PARENTERAL INFUSION OF CHEMOTHERAPEUTIC AGENTS

The delivery of chemotherapeutic agents, particularly to solid-tumor patients where treatment must continue over a substantial time, has almost always been by an intermittent, intensive schedule. The most compelling reason for this is that the drugs must be given parenterally, and it has not been practical, up to the present time, to give such drugs on a continuous basis. For some chemotherapeutic agents, schedule sensitivity has clearly been demonstrated and, were all the variables to be examined, it would probably turn out to be true for all agents. Schedule sensitivity means that the therapeutic index, that is, the toxic dose divided by the therapeutic dose, is significantly influenced by schedules of administration. The evidence that intermittent intensive treatment is superior to continuous treatment derives largely from the rapidly pro-

liferating experimental and human tumors. For methotrexate in acute lymphocytic leukemia and choriocarcinoma, and for cyclophosphamide in Burkitts' lymphoma, intermittent intensive treatment would appear to be superior. At the other extreme is the relatively slow-growing breast cancer, where comparative studies have demonstrated that continuous, ie, frequently administered, combination chemotherapy is superior to intermittent chemotherapy (9). The following factors support the position that continuous administration of chemotherapeutic agents might be more effective. Technical advances may now make such studies possible and practical in patients with solid tumors.

The Metal Bellows Implantable Pump (Infusaid) is a portable, implantable pump powered by fluorocarbon in equilibrium between gas and liquid phase (figure 3). It applies constant pressure, independent of volume, against the metal bellows. Drug is injected into the chamber and egresses through a port and catheter at a constant rate. The Infusaid volume capacity can be a few ml for experimental studies, and up to 47 ml for clinical studies. In the clinic this technique has proven effective for heparin. Here the Infusaid is implanted below the clavicle, where it superficially appears like a cardiac pacemaker. The catheter is placed in the superior vena cava through the subclavian vein. Drug is injected at 1- to 3-week intervals. This apparatus has been in place in some patients for over a year and a half and has been well tolerated (10).

As above, there is increasing evidence for patients with metastatic solid tumors that frequent drug administration may be more effective than intermittent administration. Other factors that may favor continuous administration in solid tumor patients include the following:

(a) Most chemotherapeutic agents have greater activity against mitotically active cells. The growth fraction of most solid tumors is small, but there is equilibrium between proliferating and nonproliferating cells. Thus the destruction of currently proliferating cells and those coming into cycle should be greater with continuous infusion.

(b) Many of the agents that interact with DNA (alkylating agents, most antitumor antibiotics) do so most effectively when DNA is in the process of replication. Since there may be relatively long intervals between replicative cycles in slow-growing tumors, continuous exposure offers a greater opportunity for destroying more cells.

In addition to the above general evidence that continuous administration for solid tumors may be superior, this approach might be particularly interesting for adriamycin. There is evidence that the cardiotoxicity of adriamycin may relate to peak concentrations as well as total dose. Thus several studies indicate that adriamycin, given at lower dose rates and/or with lower peak concentrations, produces substantially less cardiotoxicity (11). We are undertaking studies in animals to evaluate this hypothesis.

Continuous Infusion of Specific Drugs

Adriamycin.—Adriamycin has solubility, stability, and potency characteristics ideal for the Infusaid. We have determined in vitro that adriamycin is compatible

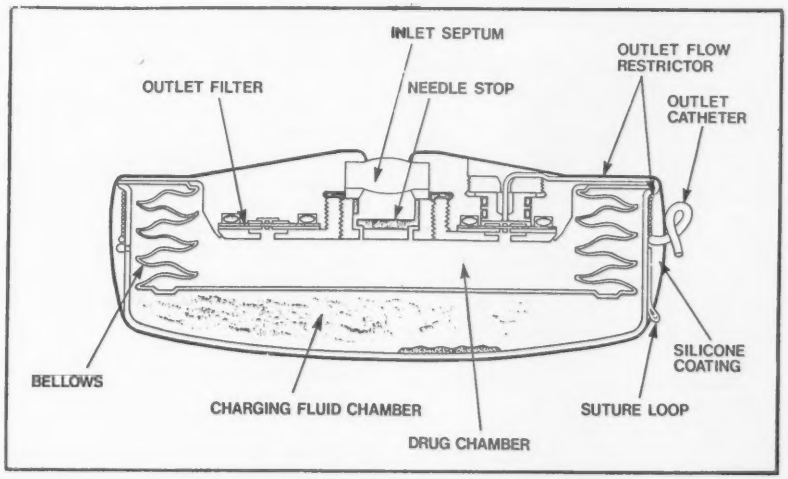

FIGURE 3.—Infusaid Model 100.

with the pump, that a constant rate of drug delivery is maintained over weeks, and that no transformation of the drug occurs as a result of interaction with the chamber. The Infusaid containing adriamycin has been implanted into the dog at our Institute and entered by external needle at 7–14-day intervals. Pharmacologic studies indicate that constant blood levels of adriamycin are maintained over a matter of several months (12).

FUDR.—Aside from the hepatic artery infusion program, FUDR has been given by bolus injection, usually in 5-day courses every 3 to 4 weeks. When given in this manner it is largely converted to 5-FU, and its activity both experimentally and in the clinic is comparable to 5-FU. On the other hand, FUDR given by continuous infusion results in a marked reduction in the tolerated dose, and it is probable that in this setting its primary biochemical effect is on DNA synthesis, in contrast to RNA for bolus injection of 5-FU or FUDR. The tolerated dose of FUDR when given by continuous infusion is markedly reduced, as compared to that when given by bolus injection. This schedule sensitivity contrasts with that of 5-FU. Moreover, there is preliminary evidence that thymidine can prevent the myelosuppressive effect of FUDR, whereas for 5-FU it tends to augment toxicity, probably by competing with degradation. It is probable, therefore, that FUDR has never been subjected to a clinical trial employing the optimal schedule (continuous infusion), except in the hepatic artery program, where response rates of 50% or 60% have been obtained.

Cis-platinum.—The nephrotoxicity of cis-platinum may relate to peak concentrations in the renal tubules. This might be substantially reduced by low-dose continuous infusion. Stability of cis-platinum is dependent upon chloride concentration, and its compatibility with the Infusaid is under study.

Other drugs.—For methotrexate, the more frequent the administration for tumors such as head and neck cancer, the greater the response. Since the ultimate frequency is continuous administration, the Infusaid might be employed. Additionally, there is recent evidence for vincristine and vinblastine that continuous infusion for 5 to 10 days might be superior to the current fixed schedule of weekly administration. Methotrexate has been found to be compatible with the Infusaid, and studies of vincristine and vinblastine are underway.

In summary, this pharmacologic technique will allow for the examination of the relative toxic and therapeutic effects of selected drugs when given by continuous infusion and provides an added dimension to clinical cancer therapeutic research.

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The Role of Target Determinants in Antineoplastic Therapy[1]

Bruce A. Chabner[2,*], Youcef M. Rustum[3], and Harry K. Slocum[3]

Given the significant toxicity and the uncertain response to most chemotherapeutic regimens, it is obvious that pretreatment prediction of response would be a highly desirable goal for antineoplastic pharmacology. The evident advances in methods for growing human explants in mice and in culture, as well as improvements in analytical techniques have greatly increased the possible range of studies that can be performed prior to treatment. However, these newer techniques must be proven useful by carefully planned prospective clinical trials, often the most complicated step in proving the value of a predictive determinant. Obvious advances have been made in the treatment of leukemias, Hodgkin's disease, Wilm's tumors, choriocarcinoma in the female, and seminoma in the male through chemotherapy, but many limitations will have to be overcome before chemotherapy can be as effective in the management of the most common solid tumors. These limitations are essentially related to the fact that anticancer drugs are generally more toxic to normal tissues, and this toxicity limits dosage. Furthermore, by unknown mechanism(s) malignant cells often demonstrate increasing resistance to drugs while normal toxicities accumulate.

In order to establish a useful in vitro system for studying the biochemical pharmacological basis of drug resistance and sensitivity of human solid tumors, certain practical problems must be overcome. They include (*a*) finding suitable methods for disaggregating cells from human solid tumors, (*b*) defining characteristics of these cells to minimize complications due to artifacts generated by the disaggregation procedures, (*c*) separating these malignant cells into subsets to define the heterogeneity of the solid tumor, and (*d*) determining whether the cell suspensions thus obtained are representative of the whole tumor. Once such a suspension is obtained it may be possible to carry out studies using human solid tumor cells for the purpose of biochemical and pharmacological characterization.

The purpose of this review is to discuss the types of biochemical studies of proven value as predictive determinants in experimental and clinical trials and to point out technical problems in procuring and processing clinical samples for such experiments.

BIOCHEMICAL DETERMINANTS IN LEUKEMIA

The range of possible determinants of drug effects is shown in table 1. These include pharmacokinetic factors (drug absorption, metabolism, excretion, and distribution) (1), host tolerance to therapeutic drug concentrations, and the kinetic and biochemical characteristics of the neoplastic cell population. The concept of population is emphasized here in order to recognize the heterogeneity of tumors as established in murine and human systems (2a, 2b). This heterogeneity adds an important element of doubt as to the possibility of making correct predictions on the basis of studies of cloned populations of cells.

In considering the biochemical determinants of drug action, it is useful to outline the steps involved in drug action at the cellular level. Some, but not all, agents encounter a significant barrier to entry at the cell membrane. While nucleosides, such as cytosine arabinoside and 5-FU, and lipid soluble drugs readily pass through the cell membrane, other agents such as methotrexate (3) and phenylalanine mustard (4) depend on active transport mechanisms. The role of drug transport in determining response has not been assessed prospectively in any human malignancy; such studies would have to be performed on a homogeneous population of

TABLE 1.—Multiple parameters of antimetabolite action

Determinants	Parameters
Pharmacokinetics (blood levels of active drug)	Biotransformation Distribution Excretion
Target cell	Transport Activation to active form Concentration of: competing normal metabolites drug-metabolizing enzymes target macromolecule or receptor

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viable tumor cells, stipulations which are difficult to meet except perhaps with human leukemic cells. It is important not to confuse transport with vaguely defined drug "uptake", which consists of a simple measurement of intracellular drug concentration after a given time period, often with no distinction between parent drug and metabolites. Such studies often confuse the accumulation of metabolized or irreversibly bound drug with a membrane-limiting event (5).

The second step in drug action is the intracellular binding to its target site, either a receptor, an enzyme, or a vital macromolecule such as DNA, RNA, or protein. This binding may require a preliminary transformation of the drug to an active metabolite, as, for example, activation to a triphosphate (purine and pyrimidine antagonists) or to a polyglutamate, a methotrexate metabolite of potential interest (6). Alternatively, drugs or their active metabolites are subject to enzymatic degradation, for example, the degradation by phosphatase of nucleotides of 5-FU (7), or 6-mercaptopurine (8) and deaminase inactivation of cytosine arabinoside (9) or adenosine analogs (10). The presence and concentration of drug receptor and the concentration of activating versus degradative enzymes are all potentially capable of determining response. Table 2 provides examples, largely drawn from studies of drug resistance in murine tumors, that illustrate the importance of each of these factors. It is clear from this table that the various studies have not reached agreement as to a single enzymatic determinant of response for common agents such as cytosine arabinoside, 5-FU, or the thiopurines. It is probable that enzymatic changes may be responsible for resistance to drugs that undergo more than one type of complex transformation.

Techniques for defining intracellular biochemical determinants include measurements of levels of intracellular enzymes in tissue homogenates (11,12), determination of the affinity of binding of the inhibitor to the target enzyme (13), or simply measurement of the intracellular concentration of the active metabolite of the antitumor agent. The latter type of determination, performed by in vitro incubation of the drug with tumor cells isolated from the host, was recently reported by Rustum and co-workers in their assessment of the relationship between ara-CTP formation and response in patients with acute myelocytic leukemia (14). Studies of the formation of active metabolites in isolated tumor cells have particular power in studying drugs that undergo complex intracellular metabolism since this measurement should reflect the net effect of a variety of possible enzymatic changes responsible for resistance or sensitivity. This type of study, however, does not identify the enzymatic changes responsible for resistance or sensitivity. Finally, it is possible to circumvent the problem of measuring a specific metabolite or intracellular enzyme by determining the effect of a drug in vitro on the activity of its target pathway, or more simply on the growth of tumor cells in culture. For example, it is possible to determine the sensitivity of tumor cells to methotrexate by examining the drug's effect on the thymidylate synthetase pathway as measured by the incorporation of deoxyuridine into DNA (15,16). Alternatively, the

TABLE 2.—Enzymatic determinants of antimetabolite action

Drug	Target enzyme	Tumor studied	Ref
Concentration of target enzyme			
Methotrexate	Dihydrofolate reductase	Human leukemia	12
PALA+	Aspartate transcarbamylase	Murine tumors	20
5-Fluorodeoxyuridine	Thymidylate synthetase	Murine neuroblastoma	21
Concentration of degradative enzyme			
Ara-C	Cytidine deaminase	Human myelocytic leukemia	9
Ara-A	Adenosine deaminase	Murine leukemia	22
6-Thiopurines	Alkaline phosphatase	Human acute leukemia	23
5-FU	?Phosphatase		7
Concentration of activating enzyme			
Ara-C	Deoxcytidine kinase	Human acute leukemia Murine L1210	11
6-Thiopurines	Hypoxanthine-guanine phosphoribosyl-transferase	Murine leukemia	25
5-FU	Multiple enzymes	Murine leukemia	26

drug may be incubated with cells in vitro prior to growing them in culture, and effects on clonogenicity must be determined. The first type of experiment has not been successfully applied to predicting response in man with the possible exception of studies of methotrexate inhibition of nucleoside incorporation into DNA of AML cells (15,17). It is difficult to duplicate in vitro the many biochemical and pharmacologic conditions that modify response in vivo, including drug concentration, duration of exposure, concentration of rescue metabolites, and drug interactions in combination regimens (18). Experiments that depend on inhibition of tumor colony growth in vitro are subject to the same limitations with the additional disadvantage that one must wait 7–14 days for an answer in order to be useful clinically and must be able to grow the tumor in question reliably (19). In general, studies utilizing whole cells in vitro are more likely to be successful with drugs like nitrosoureas, alkylating agents, and antibiotics since these agents are less affected by the modifying conditions listed above and require only brief exposure durations to exert their cytotoxic effects.

Elsewhere in this issue, specific approaches to the prediction of response for the various classes of antineoplastic drugs will be discussed. The vast number of possibilities for such studies, as discussed above, is further complicated by the problem of designing a prospective study in man. Since most responsive tumors in man are treated by a combination of agents, it is usually impossible to ascribe response or remission to the action of a single agent. A failure to respond is a clear-cut indication of insensitivity to all agents used, although deaths during induction of acute leukemia may be due to infection, hemorrhage, or other complications and may have little to do with drug sensitivity. Thus the clinical test of a potential determinant may present serious problems due simply to the difficulty of designing and interpreting an appropriate clinical trial.

BIOCHEMICAL DETERMINANTS IN HUMAN SOLID TUMORS

In spite of intensive effort on the part of many investigators in the past 30 years, no effective in vitro test to predict the chemotherapeutic reactivity of human solid tumors is yet established. Tests involving the use of slices or minces of tumor materials (table 3) suffer from three basic problems: (*a*) multiple cell types including normal, stromal, and heterogeneous tumor cell populations obtained in slices and minces are bound together; (*b*) cells found deep in a tissue piece are in a different microenvironment as regards access to test medium, so cells in every way identical except location could be expected to react differently in any test utilizing solid pieces of tissue; and (*c*) different pieces or slices may contain different populations of cells in varying proportions, thereby making it impossible to compare tests run on different aliquots of the same preparation.

TABLE 3.—Problems related to study of solid tumors

Heterogeneity
Physical position of cell in tissue during study
Normal, stromal, and malignant elements mixed in varying proportions
Viability variation dependent on distance from vasculature
Target cells not anatomically segregated
Studies precluded by solid form of tissue
Viability assessment
Cell separation for characterization—drug uptake rates on a cellular level, clonogenicity, flow cytometry

The production of single cell suspensions from solid tumors for biochemical and pharmacological studies will remedy these problems but has a number of critical requirements. First, cell yield must be maximized. If only a small proportion of cells in the solid tumor are present in the cell suspension obtained, chances are markedly reduced that they represent all important populations within the tumor and, indeed, the target cells one wishes to characterize may be entirely absent. Also, cell yield must be maximized because limited amounts of human material are available at biopsy and many studies must be carried out using the cell suspension obtained in order to perform the important biochemical and pharmacologic tests on an individual tumor.

Two basic approaches have been used in producing single-cell suspensions: mechanical and enzymatic tissue dispersal (27,28), as outlined in table 4. Mechanical disaggregation has been used by many groups (29,30) because it has the advantages of speed and simplicity and avoids the complication of serious (and possibly subtle) effects of enzymes on cells and their surfaces. The stresses of mechanical dispersion on cells, however, may often result in poor yields (31,32), and suspensions produced mechanically often contain large numbers of dead cells (33). Unfortunately, quantitative data concerning cell yield are seldom available, and this makes assessment of procedures dif-

TABLE 4.—Methods for disaggregation of human solid tumors

Mechanical disaggregation
Advantages: speed, simplicity, freedom from subtle artifact
Problems: poor yields, contamination with dead cells
Enzymatic Disaggregation
Advantages: high yields, high "viability"
Problems: uptake of enzyme, cell membrane changes

ficult. Enzymatic procedures for the production of cell suspensions from solid tissues have long been in use for establishing primary cell cultures. These procedures, however, have often proven to be specific for a particular tissue and even within a particular tissue type may be successful only for a particular subclass of cells. Also, there is disagreement as to the effect of enzymes on cells. For example, collagenase has been reported to be innocuous (34,35) or harmful (36–38). Likewise, pronase has been reported to be deleterious (39) and nondeleterious (40–42). These discrepancies are likely due to differences in tissues, media, and conditions applied, and may also be due largely to the fact that minor enzyme activities contained in crude preparations of enzymes are often important contributors to toxicity and disaggregation; thus different batches of enzyme preparations may vary considerably in activity. Fortunately, disaggregating activity is sometimes separable from toxic activities by partial purfication of the enzyme (36).

Enzymatic approaches in human solid tumors have been used to disaggregate human brain tumor (43), breast (35), and prostatic carcinoma (44,45), Hodgkin's disease (46,47), colon carcinoma (48,49), and a variety of other carcinomas (50). Mechanical and enzymatic methods of producing cell suspensions from human melanoma, sarcoma, and lung tumor specimens have been evaluated. Mechanically produced cell suspensions were generally characterized by yields of less than 20×10^6 dye-excluding cells/g of tissue, with large numbers of nonviable cells included in the suspension. While various enzymes and conditions were examined initially, the best method consisted of slicing the solid tumor with a Stadie-Riggs microtome and incubating these slices with 0.8% collagenase II (Worthington) and 0.002% DNAse I at 37° for 2 hours. By this method, yields exceeding 20×10^6 dye-excluding cells/g and substantially free of Trypan blue staining cells (15% or less) could be obtained in a majority of cases. In this manner, cell suspensions were ready for study within approximately 3 hours of surgical removal of specimens.

Methods for releasing large numbers of cells from solid tumors are, of course, only the first requirement for solving the problem of sampling the most common human malignancies. The next question to be addressed concerns characterization of cells released. What fraction of the cells comprising the tumor are released by any given method? Are important populations being destroyed or omitted? Are the cells obtained by a given method seriously altered, especially in regards to critical determinants of drug responsiveness?

We have characterized cells obtained by various methods as to incorporation of RNA, DNA, and protein precursors, ribonucleotide pool sizes, enzyme profiles, and cloning in soft agar.

To summarize our preliminary results, precursor incorporation has been universally low. Enzyme activation related to nucleoside and nucleotide metabolism has been variable, but generally appears in all suspensions to be similar to the original tissues. Ribonucleotide pool profiles have been variable in some cases but usually are very consistent with pools in enzymatically released "healthy" cells, though lower in mechanically released suspensions. Except in certain types of lung carcinoma, clonogeneity in soft agar has generally been better in enzymatic than mechanically released suspensions. Thus these measures of functional integrity generally have revealed no major damage to the cells released by the enzymatic method. In additon, treatment of animal model tumors by this method has not reduced regrowth potential upon reimplantation of treated cells into the host (Slocum and Rustum, unpublished results).

The investigation of human solid tumors at the cellular level requires that adequate disaggregation techniques be applied initially and the cell suspension so produced be well characterized. Thus major methodological improvements may be required before the complex questions of tumor cell biology and pharmacology can be answered for solid tumors. Cell separation techniques must then be developed or modified from current methodology to answer the important questions of tumor cell biology and pharmacology that will allow understanding and prediction of therapeutic responsiveness of individual patients.

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Advantages and Limitations of Pharmacokinetic Studies in the Rationalization of Anticancer Therapy: Methotrexate and 5-FU[1]

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SUMMARY

The use of pharmacokinetic studies for individual dose regimen adjustments in anticancer therapy is considered. The example of methotrexate in the treatment of head and neck tumors demonstrates the validity of this approach. Moreover, the importance of biotransformations of this antimetabolite is confirmed using a high pressure liquid chromatography assay. The example of 5-FU outlines the analytical and mathematical difficulties rendering this approach unlikely for routine use. Some preliminary relations between pharmacokinetic parameters (plasma clearance) and clinical response are presented. The determination of the main plasma metabolite 5-6 dihydro 5-FU by GC/MS and its possible role in the nonlinear pharmacokinetics of the drug in therapeutic failure are discussed.

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The main goal of pharmacokinetics is to design and experimentally validate mathematical models describing the time course of drug concentration in the body (1,2). These models have been developed in roughly two different directions (3).

One model relies directly on physiological and anatomical bases, including phenomena such as blood flow, membrane transport, enzymatic reactions. The other, simplistic, model mimics or fits the available experimental data (generally plasma and urinary concentrations). This approach can be extended somewhat by introducing hypothetical compartments in which the drug is presumed to be homogeneously distributed, leading to the so-called compartment systems.

A number of reasons can justify the development and the use of physiological models. These models allow the predictions of concentrations in organs and tissues (3–6) and consequently are of appreciable help in rationalizing toxicity studies. They are also adaptable to changing physiological circumstances: for instance, changes in blood flow or renal clearance. But in spite of these benefits, this kind of model presents some shortcomings (3).

Since physiological models usually introduce a large number of parameters, much data are required for their design and validation. Moreover, these data can be difficult to obtain, especially when working with humans. Also, these models are often composed of a set of complex nonlinear differential equations, requiring extensive numerical computation facilities. For all these reasons, the use of these physiological models is very difficult, at least for routine purposes. (3)

In contrast, compartment models, and more specifically linear compartment models, introduce the minimum number of parameters to fit accurately enough the experimental data. So even if they lack a direct physiological interpretation, they are easier to handle mathematically and numerically and are consequently highly suitable for clinical applications, as long as the blood or urinary concentrations of the drug can be considered as reflecting relevant pharmacologic and toxic properties of the drug.

Let us recall two basic assumptions for these models (1,2). The transfer rate of the drug between the various compartments is proportional to the concentration in

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the original compartment (hypothesis of linearity), and all parameters such as volumes or rates are assumed to remain constant during the entire observation period (hypothesis of stationarity).

Finally the success of this type of modeling is based on the existence of a complete mathematical theory describing the behavior of drug concentrations after any classical mode of administration and allowing the estimation of the unknown parameters from experimental data. But it must be stated that the use of these linear compartment models implies that one has already performed experiments to prove that the hypotheses of linearity and stationarity are reasonably valid.

The aim of this paper is to illustrate how compartment models can be used in real clinical situations for the individual dose regimen adaptation of anticancerous drugs. The first example concerns high dose methotrexate (MTX) in the treatment of head and neck cancers. This drug exhibits a linear behavior, in the usual therapeutic range at least, and therefore a protocol can be proposed to understand the interindividual variations in the kinetic response. The second example deals with 5-FU in the treatment of gastrointestinal tumors. Here the analytic difficulties, combined with the obvious nonlinear kinetics of the drug lead to a more complicated situation. While an individual adjustment of dose regimen cannot yet be proposed, a possible approach including the determination of the main plasma metabolite of 5-FU will be presented.

METHOTREXATE

High-dose MTX with leucovorin rescue is widely used (7), but it is well known that this antimetabolite results in toxic reactions that can be attributed not only to the dose but also to the duration of MTX exposure (8,9).

When we administered 1.5 g/m² of MTX during a 36-hour continuous infusion, we observed significant individual variations in the plasma concentrations of the drug during and after the infusion. Some patients exhibited undesirable reactions (mostly buccal ulcerations). To reduce the frequency and degree of those toxic manifestations we proposed individualization of the MTX dose so all patients could achieve a 10^{-5}M steady-state plasma level of the drug. This requires determination of the kinetics of unchanged MTX in each patient (10).

Protocol with Pharmacokinetic Identification

The proposed protocol consists essentially of three successive phases as outlined in figure 1.

1. Pharmacokinetic characterization of the patient after a moderate test-dose of MTX (50 mg/m²); plasma determinations are performed using a protein binding assay (11);

2. Estimation of pharmacokinetic parameters and computation of a MTX dose to ensure a plasma level of 10^{-5}M during the 36-hour infusion;

3. Administration of the infusion and therapeutic monitoring with enzymatic assay (12).

Theoretically, the most relevant pharmacokinetic parameter for this study is total body clearance (Cl), defined as the volume of the central compartment from which the drug is removed before being eliminated from the organism, per unit of time. This parameter, constant under the hypotheses of linearity and stationarity and for any intravascular mode of administration, is given by the simple formula

$$Cl = \frac{\text{Dose}}{\text{AUC}}$$

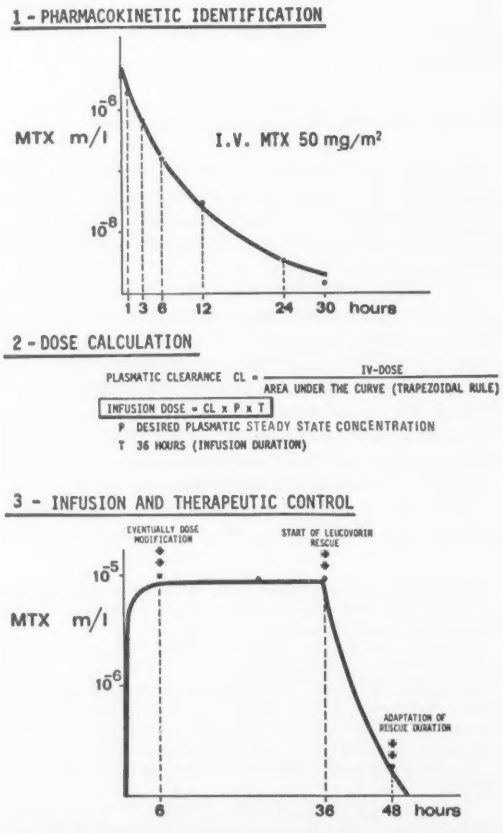
1 - PHARMACOKINETIC IDENTIFICATION
MTX m/l
10⁻⁶
10⁻⁸
I.V. MTX 50 mg/m²
1 3 6 12 24 30 hours
2 - DOSE CALCULATION
PLASMATIC CLEARANCE Cl = IV-DOSE / AREA UNDER THE CURVE (TRAPEZOIDAL RULE)
INFUSION DOSE = Cl x P x T
P DESIRED PLASMATIC STEADY STATE CONCENTRATION
T 36 HOURS (INFUSION DURATION)
3 - INFUSION AND THERAPEUTIC CONTROL
EVENTUALLY DOSE MODIFICATION
START OF LEUCOVORIN RESCUE
MTX m/l
10⁻⁵
10⁻⁶
ADAPTATION OF RESCUE DURATION
6 36 48 hours

FIGURE 1.—Principal phases of the personalized MTX protocol.

where AUC denotes the area under the plasma concentration curve.

The total body clearance characterizes the steady-state level during a long term continuous constant rate of infusion. The plateau level (P) at this steady state level is given by:

$$P = \frac{\text{Rate of infusion}}{\text{Clearance}}$$

Therefore, once the clearance of a patient has been estimated in preliminary tests by administration of an MTX test-dose, if one desires a given steady state plateau level one can then compute the corresponding dose to be administered to a given patient (fig 1). This is the basic principle of the individual adjustment protocol.

Results and Comments

So far about 60 patients have been treated according to this protocol. Taking into account the large number of possible errors, we find good agreement between the experimental results and the mathematical predictions. During infusion the desired plasma level was achieved in most patients, and a significant reduction of side effects was noted. In this clinical pharmacokinetic study, the plateau level ($P = 10^{-6}M$) was chosen arbitrarily.

In some patients, however, we noticed wide discrepancies between mathematical predictions and experimental data. Of the various possible explanations, we retain two: (*a*) nonspecificity of the enzymatic assay used during therapeutic monitoring, due perhaps to potential interferences of the main plasma metabolites of MTX (7 OH-MTX, DAMPA) (14,19) as well as to other exogeneous or endogeneous substances; (*b*) possible nonstationarity of the model, which would invalidate the test-dose approach.

Analytical Considerations

Figure 2 represents the pharmacokinetics of MTX given in an iv bolus test dose. After determination of total body clearance, a MTX dose of 1.86 g was computed and administered for 36 hours (fig 3). Drug monitoring using the enzymatic assay showed that the MTX plasma level increased almost continuously during the infusion. With a more specific technique such as high pressure liquid chromatography (HPLC) (13), it can be seen that the unchanged MTX level remains constant and close to the desired level of $10^{-5}M$.

This example illustrates the fact that therapeutic individualization of high-dose MTX using a specific method remains pharmacokinetically valid.

Metabolism Incidence

Another point of particular interest concerns the importance of MTX metabolism (14). Using the protein binding assay, significant interpatient variations in the total body clearance of MTX (58–360 ml/min) were observed during the pharmacokinetic characterization

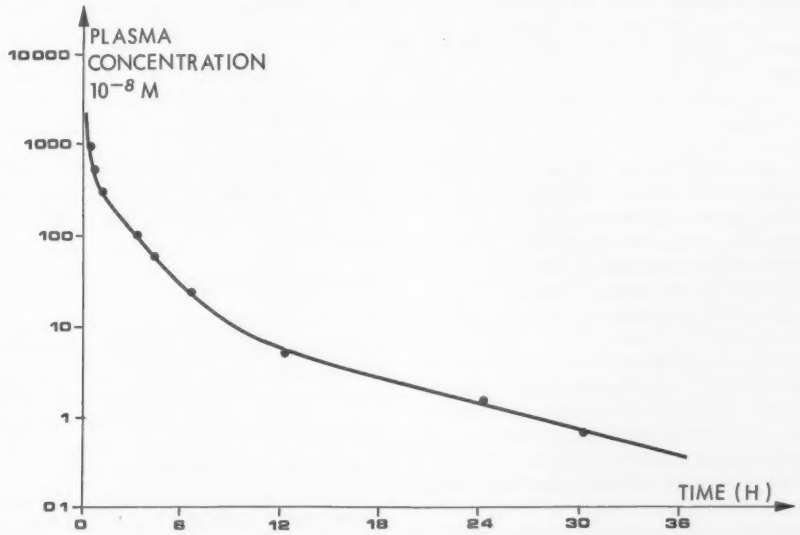

FIGURE 2.—Time course of MTX plasma level after iv bolus of 85 mg. ● = experimental data; —— = computer calculated values.

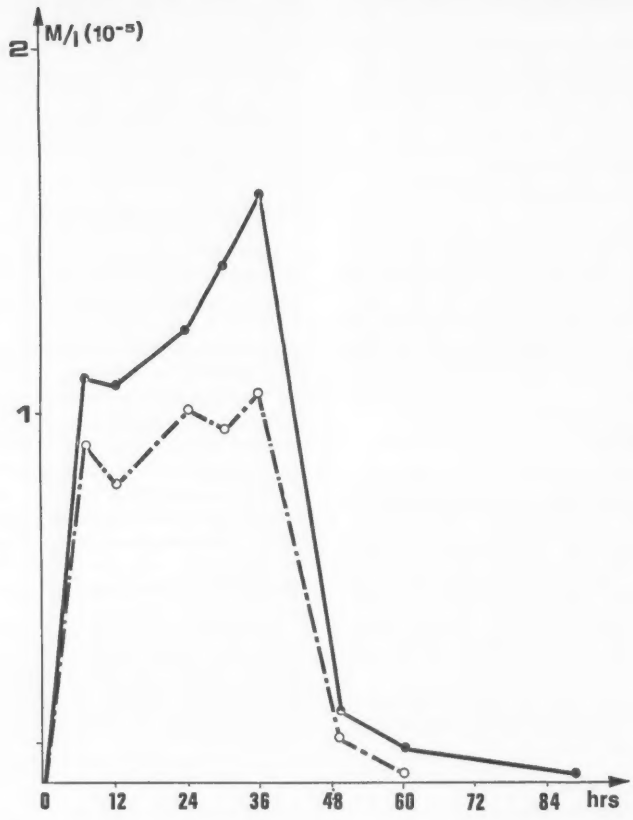

FIGURE 3.—MTX plasma levels during 36-hour infusion. ●——● = enzymatic assay; ○——○ = high pressure liquid chromatographic assay.

of the patient. The highest values suggest a significant hepatic clearance or metabolism. 7-OH MTX has been found in urine and plasma of patients and is likely to be the main metabolite after high dose drug infusion (14–16). As was recently suggested by Donehower et al (16), 7-OH MTX and the glutamate cleavage product are much less water-soluble than the parent compound. It is important to confirm if interpatient variation in MTX metabolism can partially explain renal toxicity.

As an example, figure 4 shows the urinary elimination of MTX and 7-OH MTX in one patient during a 36-hour infusion. As can be seen, 30% of the dose is eliminated as 7-OH MTX and 50% as unchanged MTX.

A very specific assay such as HPLC (17–21) for drug monitoring during the therapeutic phase therefore permits measuring the actual level of unchanged MTX. Moreover, a metabolic approach could help in categorizing patients who might have an extensive hepatic clearance. Thus the extension of pharmacokinetic identification, not only in plasma but also in urine, should allow the benefits and the risks of such a therapy to be estimated.

5-FLUOROURACIL

It is fairly easy to propose pharmacokinetically based protocols for dose regimen adjustment when the drug presents linear kinetics and when the analytic imperatives are not too complicated. However, things can become more tricky when dealing with nonlinear kinetics and sophisticated assays. These two difficulties are encountered with 5-FU.

Nonlinear Kinetics

With 5-FU it was necessary to develop highly sensitive and very specific methods such as gas

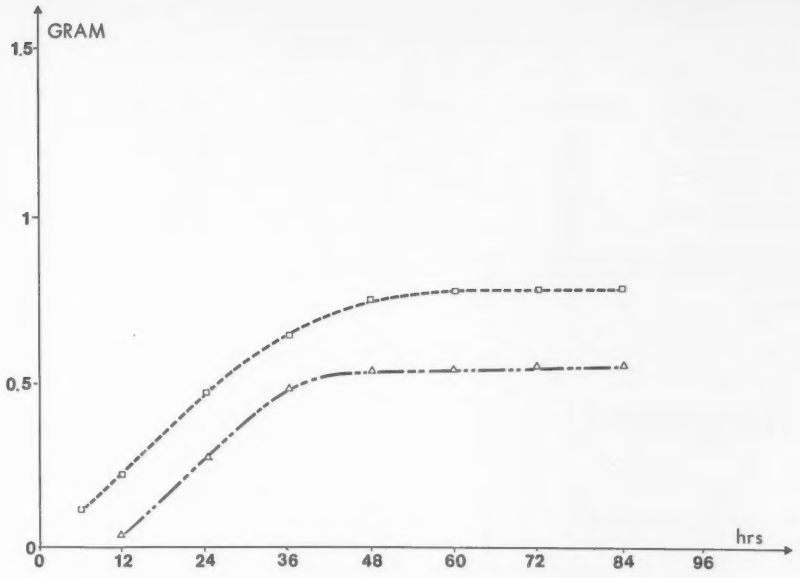

FIGURE 4.—Time course of cumulative excretion in urine after 36 hours MTX infusion 1.620 g. □····□ = MTX HPLC assay; △····△ = 7-OH MTX assay.

chromatography coupled with mass spectrometry (GC-MS), to determine the plasma kinetics of the unchanged compound (22–28). After an intravenous bolus of 15 mg/kg of 5-FU, the time course of plasma concentrations exhibited a very rapid decrease within the first 2 hours. Interestingly, we have detected in some patients a slow elimination phase 2–4 hours after drug administration (fig 5).

In a previous paper (28), we documented the nonlinearity of the unchanged 5-FU kinetics. Table 1 shows total body clearance values after both iv bolus and 8-hour infusion. It can be seen that the clearances obtained after these two modes of administration differ by factors of 10-40 even though this parameter should remain constant under the hypothesis of linearity.

In a few patients we studied the influence of infusion duration for the same dose (15 mg/kg) on the total body clearance (table 2). Figure 6 illustrates the variation of this parameter plotted against the reciprocical of the infusion flow rate. We did not find any plateau in this curve, with infusion length up to 6 hours, although one could reasonably expect that for such a duration the kinetics would behave almost linearily.

Relations Between Pharmacokinetic Parameters and Clinical Response

We tried to find eventual relations between some pharmacokinetic parameters and the clinical response based on the tumor volume (29). Twelve patients with advanced digestive cancers (8 colorectal, 2 unresectable pancreatic carcinomas, 1 carcinoid tumor of the small bowel with pancreatic metastasis, and 1 hepatocholangiocarcinoma) were admitted into a two group randomized study. Group A received 5-FU via 15 mg/kg/week iv bolus, and group B via 15 mg/kg/week 8-hour infusion. For pharmacokinetic purposes, group B patients also received a 15 mg/kg iv bolus of 5-FU prior to the initiation of therapy.

Among the eight colorectal cancer patients there was one partial response, and four patients showed disease stabilization but no response. For the four patients with other types of digestive cancers, only one was stabilized. It is interesting to compare the total body clearance values (following iv bolus administration) with clinical response based on tumor growth, for the duration of 3–15 months. It seems (fig 7) that partial response or stabilization occurred with patients who exhibited a very low clearance after iv bolus administration (ie < 0.8 liter/min). There was only one exception, a patient who was stabilized after treatment in spite of the fact that his clearance was very high (1.77 liter/min). In this case, the stabilization could be due to the nature of the malignancy (pancreatic cancer of slow evolution) rather than to the effect of the treatment. In a certain sense, these remarks are similar to Hillcoat's observation (30,31) of a correlation between tumor growth and area under experimental data (C x t values).

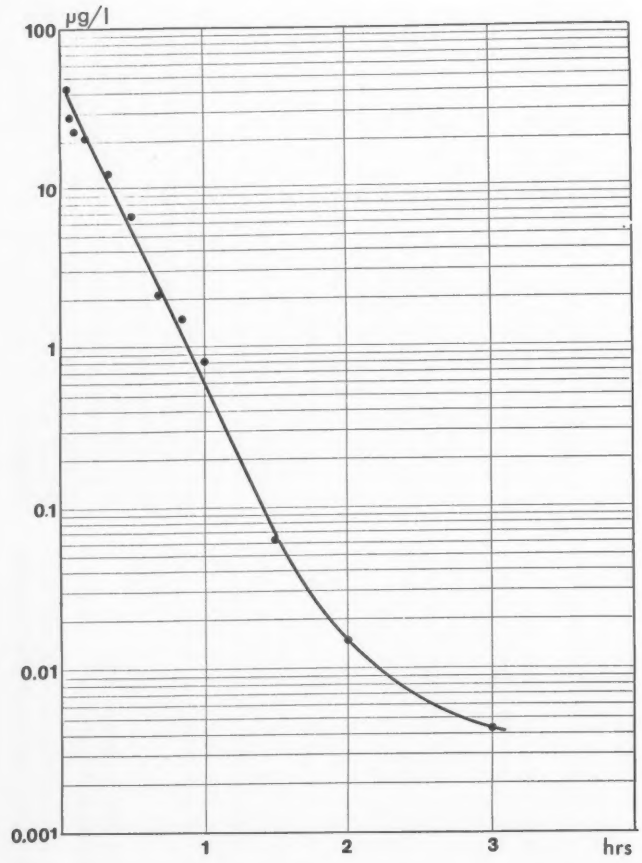

FIGURE 5.—Time course of 5-FU after iv bolus showing a slow elimination phase.

TABLE 1.—Plasma clearance values of 5-FU after iv bolus and 8-hour infusion

Patient		Dose (mg)	Iv clearance l/min	Infusion (8 hrs) clearance l/min
Gon	1	750	0.39	8.35
Chi	2	900	0.62	
Mei	3	885	1.32	
Tom	4	700	0.86	
Mou	5	1000	0.56	14.5
Bro	6	1000	0.72	27.5
Gui	7	550	0.65	
Den	8	1155	2.55	
Tru	9	600	1.32	
Coe	10	800	1.04	
Mar	11	1100	1.77	
Dai	12	700	1.47	58.0
Mean		—	1.11	27.1
Standard Deviation of mean (s = 0.95)		—	0.38	—

TABLE 2.—Kinetic parameters of 5-FU after variable length infusions

Name, dose (mg)	Infusion Duration	Plateau level (μg/ml)	Clearance L/min	Clearance determination methods
800	< 2 min	—	1.04	Area under experimental curve
800	15 min	—	1.74	
Coe. 800	2 hr	~1.4	4.76	Plateau level
800	6 hr	~0.2	11.11	
600	< 2 min	—	1.32	Area under experimental curve
Tru. 600	30 min	—	1.78	
600	3 hr	~0.6	5.6	Plateau level

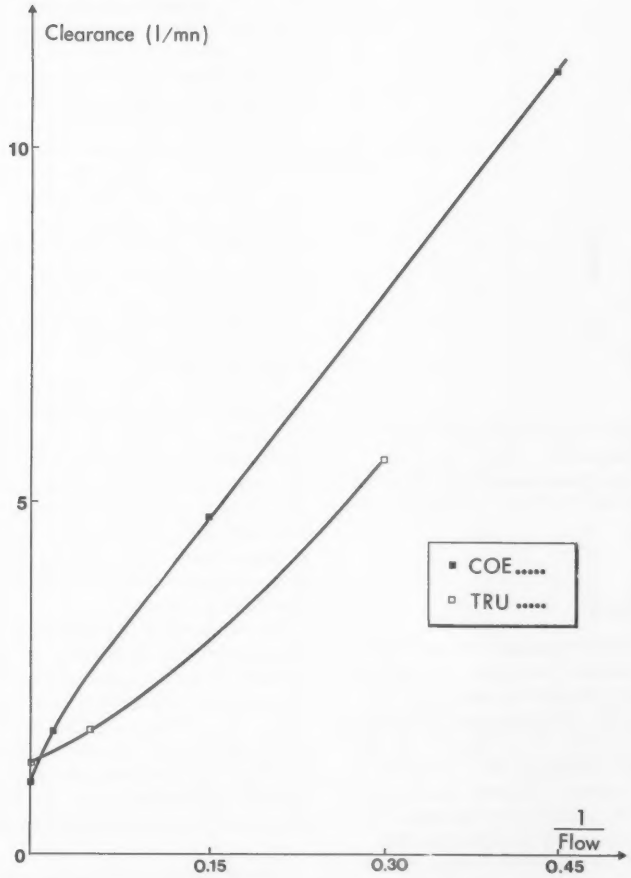

FIGURE 6.—Clearance variations versus the reciprocal of the infusion flow rate.

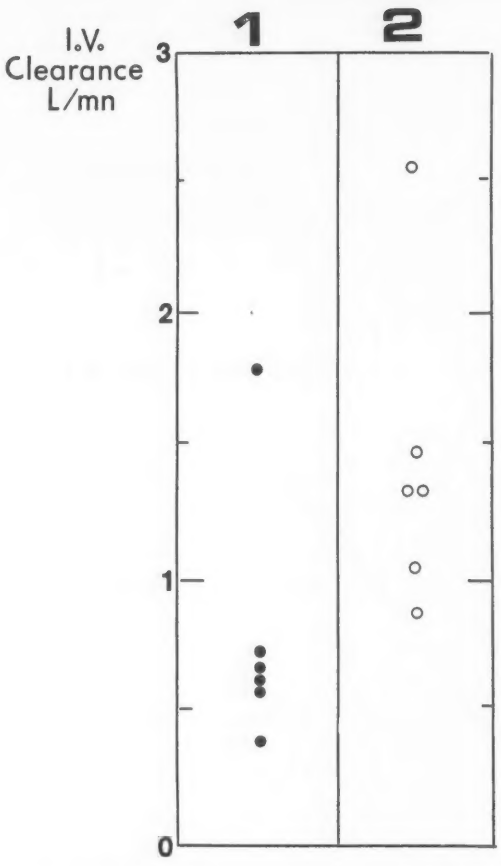

FIGURE 7.—Correlation between plasma clearance (iv bolus) and clinical response, stabilization or partial response (1) and nonresponse (2).

Role of 5,6 Dihydro 5-FU

Various authors (32–34) have pointed out the importance of the catabolic pathway to explain therapeutic failures with 5-FU. Figure 8 depicts the processes of activation (anabolic pool) and of degradation (catabolic pool) of 5-FU. Since anticancer action depends on the balance between these two processes, the catabolism of 5-FU in human cancer patients "is a contributing factor in setting up a clinically effective dosage regimen," as was claimed by Chaudhuri and Heidelberger (32).

In order to explore the possible origin of the nonlinear 5-FU kinetics, it seemed very important to us to determine the main plasma metabolite of 5-FU, which is supposed to be 5,6-dihydro-5-fluorouracil (5-FU-H_2).

We purified 5-FU-H_2 by HPLC, then set up a new GC-MS assay to allow the quantitative plasmatic determination of this major metabolite. This quantification is performed by monitoring with a data system the base peaks at M/e 160 and M/e 154 following extraction with isopropanol/ether (25:75, V/V) and derivatization with iodomethane using thymidine as an internal standard.[4] As an example, we have reproduced in figure 9 the time course of plasma concentrations of 5-FU and 5-FU-H_2. The values obtained confirm the importance of the circulating levels of 5-FU-H_2.

CONCLUSION

This paper brings out two imporant points. The first one is the possible use of pharmacokinetic studies for individual dose regimen adjustment in everyday clinical practice. When the kinetic model is simple enough (linear model) and when the assays available are not too complicated, this approach is valid and leads to an obvious improvement in the mastering of plasma levels during the therapeutic phase and hence in the control of toxic side effects. On the other hand, this approach can fail when either the analytical methods are too sophisticated or the kinetic model is too complex, or both. We are not yet in a position to propose an individual adaptation of 5-FU therapy, and if we have solved the problem of a pharmacokinetic model design for this agent, we think that the application of such an approach to routine clinical practice would be unlikely.

The second point concerns the importance of metabolic studies in the clinical pharmacokinetic field. In the case of MTX, the determination of 7-OH MTX could permit a prediction of nephrotoxicity. For 5-FU, the measure of 5-FU-H_2 in plasma could give some insight into the causes of nonlinear kinetic behavior and into the possible origins of some therapeutic failures. It is interesting to note that in this case the determination of an inactive metabolite is useful for clinical pharmacology purposes.

We recommend the following lines of research: (*a*) selection of the best route of administration of anticancer drugs; (*b*) exploration of the mechanism of interactions between MTX and 5-FU in order to determine, for instance, whether MTX is likely to change the production of dihydrofluorouracil; (*c*) research for new fluoropyrimidines able to bypass the generation of inactive metabolites such as 5-FU-H_2 (one of these drugs could be 5'-deoxyfluorouridine).

[4]Aubert C, and Cano JP. Simultaneous determination by CG/MS of 5-fluorouracil and its main metabolite (5,6 dihydro 5-fluorouracil. To be published.

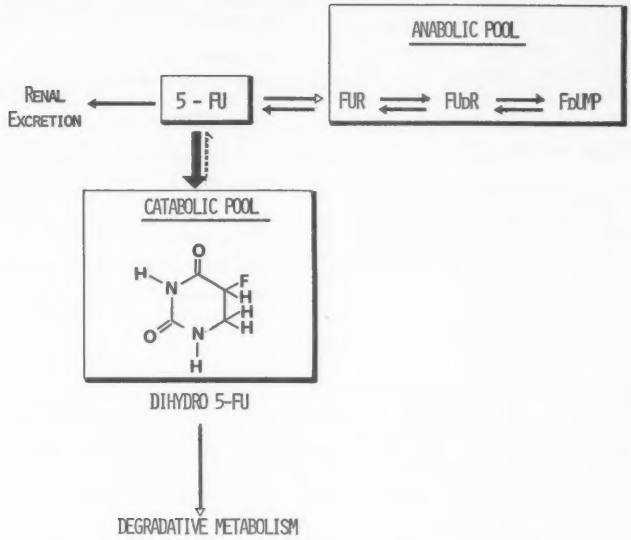

FIGURE 8.—Processes of activation (anabolic pool) and of degradation (catabolic pool) of 5-FU.

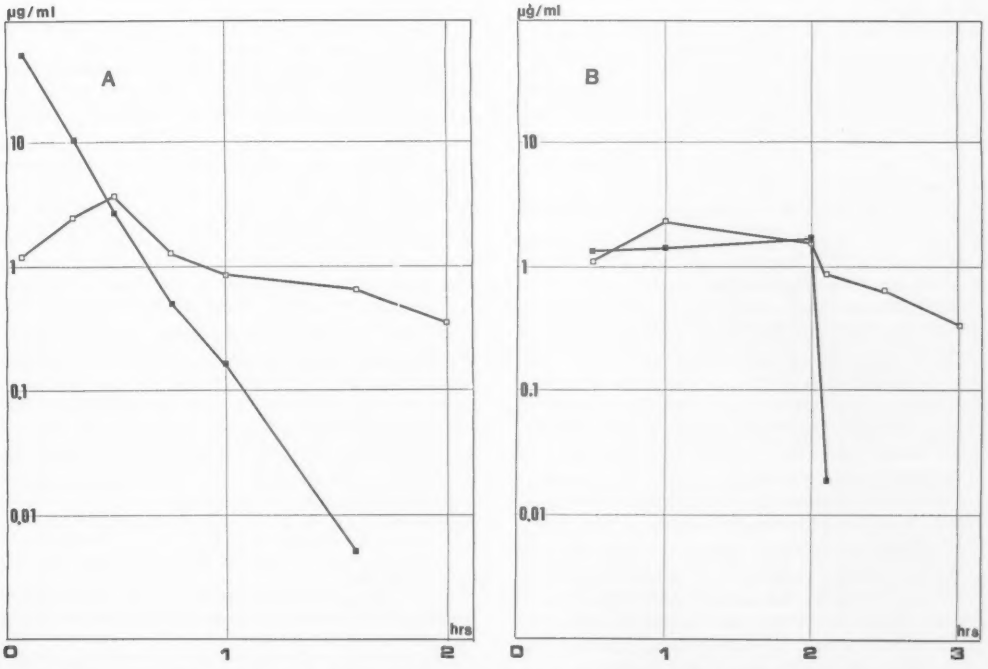

FIGURE 9.—Time course of 5-FU and 5,6 dihydro 5-FU plasma levels, following iv bolus (A) and 2-hour infusion (B), in the same patient (15 mg/kg).

In conclusion, it should be emphasized that pharmacokinetics must include studies not only of drug disposition phenomena but also of the clearance modalities, including metabolism. Indeed, drug efficiency and drug toxicity are closely related to the metabolic pathway of the agent, which is furthermore of utmost importance when dealing with antimetabolites.

It would seem that the best way to progress in clinical pharmacology of anticancer drugs is to associate good methodology (highly sensitive, specific, and reproducible) with comprehensive mathematical treatment of the available data.

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Some Considerations in the Experimental Approach to Distinguishing Between Membrane Transport and Intracellular Disposition of Antineoplastic Agents, with Specific Reference to Fluorodeoxyuridine, Actinomycin D, and Methotrexate

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SUMMARY

Analysis of the interaction between an anticancer agent and the intact cell can provide important clues to cytotoxic determinants and mechanisms of drug selectivity and resistance. This can be very useful in optimally evaluating single and multidrug regimens in test systems, designing new agents, and evaluating drug metabolism and binding to cellular target sites. This cellular pharmacokinetic approach requires very meticulous attention to experimental design, especially the time dimension chosen for study, and an appropriate framework for data analysis. This paper reviews cellular pharmacokinetic principles and experimental approaches that can be applied in "uptake studies" with cytotoxic agents, focusing, as examples, on studies from this laboratory with fluorodeoxyuridine, actinomycin D, and methotrexate.

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Various phases of the time-course of uptake of a cytotoxic agent, or its radiolabeled derivatives into mammalian cells reflect the interaction between the agent and (*a*) the cell membrane, (*b*) a membrane transport carrier, (*c*) intracellular enzyme site(s) that result in its metabolic conversion (often to active derivatives), (*d*) other intracellular sites to which the agent binds or reacts chemically (that may result in its pharmacologic activity), or (*e*) the membrane of an organelle through which the agent or its derivatives penetrate that may contain an interactive site.

Distinguishing phenomena related to membrane transport from those related to binding, metabolism, or compartmentation in the uptake of an anticancer agent is critical to defining elements of the drug-cell interaction that may play an important role in cytotoxicity, selectivity, and tumor resistance. These considerations may be extremely useful in optimizing single or multidrug regimens, the design of new agents, and the evaluation of drug metabolism and binding within the cell. This investigative approach requires very meticulous attention to experimental design, especially the time dimension chosen for study, and an appropriate framework within which uptake data are interpreted.

There is often considerable difficulty in discriminating between uptake that reflects membrane transport and uptake that reflects some subsequent intracellular event. For instance, analysis of uptake data from cells resistant to an agent due to a defect in metabolism could lead to the erroneous conclusion that there is a transport deficiency. Conversely, alterations in cell sensitivity due to a primary change in membrane transport resulting in decreased availability of the drug to intracellular sites might be erroneously attributed to a primary defect in a metabolic pathway. Accordingly, distinction between these different elements of the drug-cell interaction is essential.

Analysis of the membrane transport of an anticancer agent cannot be undertaken without a broader view of the nature of the interaction between the agent and the intact cell. Likewise, analysis of the kinetics and control of drug metabolism, binding, and compartmentalization within the cell requires an understanding of the kinetics and properties of the membrane transport process. These phenomena are explored by applying cellular pharmacokinetics, a highly quantitative ap-

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proach that evaluates many elements in the interaction between the drug and the intact cell, and the effects of this interaction on the cellular metabolic pathways that the agent perturbs, ie, the nature of the drug-cell interaction resulting in a pharmacologic effect and cytotoxicity.

This paper reviews principles of cellular pharmacokinetics employing as examples studies from this laboratory relating to several classes of compounds: (*a*) FdUrd[3], an agent that is rapidly metabolized to active derivatives retained within the cell as well as products that rapidly leave the cell (1), and deoxyuridine, another rapidly metabolized nucleoside (2), (*b*) actinomycin D, an anthracycline that achieves its pharmacologic effect by binding to its intracellular target sites (3), and finally, (*c*) methotrexate, an agent that while binding very tightly to its target enzyme, dihydrofolate reductase, is ordinarily a very rapidly reversible inhibitor of tetrahydrofolate synthesis within the cell. This paper will, however, focus on recently explored aspects of methotrexate pharmacology in the isolated rat hepatocyte (4,5) that relate to binding of this agent to sites other than dihydrofolate reductase and its rapid conversion to polyglutamate derivatives, a metabolic step with the potential to decrease the reversibility of methotrexate inhibition of dihydrofolate reductase activity within the cell.

CELLULAR PHARMACOKINETICS OF FdUrd WITH A BRIEF REFERENCE TO DEOXYURIDINE

The cellular pharmacokinetics of FdUrd illustrate many important principles that must be considered when evaluating uptake of a cytotoxic agent that is metabolized within the cell to derivatives that do and do not penetrate the cell membrane (1). A number of key elements of the drug-cell interaction and the experimental approaches employed for their study are reviewed. Other papers have recently been published relating to the distinction between transport and metabolism of nucleosides in mammalian cells 6–8).

Defining the Initial Uptake Velocity: Analysis of the "Zero Time" Ordinate Intercept

The critical parameter of the drug-cell interaction that clearly delineates membrane transport from any subsequent intracellular event is the initial uptake phase. The drug, usually radiolabeled, is added to a cell suspension and cell radiolabel is measured as a function of time. If this relationship is shown to be linear, it is usually assumed that it represents the initial flux of the drug into the cell. That is, the interval over which uptake of radiolabel into the intracellular compartment is unidirectional and there is negligible backflux. Critical to the interpretation of these data is the time frame over which the experiment is performed and a quantitative analysis of the level of radiolevel associated with the cell relative to its extracellular concentration. Identification of an early linear uptake phase does not ensure that this represents membrane transport.

Figure 1 illustrates the time-course of ^3H uptake 20 to 100 seconds after exposure of Ehrlich ascites tumor cells to 0.2 μM [^3H]-FdUrd. Uptake over this interval is linear and might therefore be interpreted as a unidirectional flux of [^3H]-FdUrd into the cell. Of particular interest, however, is that when the uptake slope is extrapolated to zero time, it intercepts the ordinate above the point of origin, indicating a rapid association of ^3H with the cells. Such an intercept is frequently attributed to an instantaneous adsorption of drug to the cell surface, complete by the time the first uptake measurement is made.

Often, it is not possible to detect a rapid uptake component of this magnitude at all when the time dimension over which uptake is monitored is longer. For instance, by 100 seconds, the ordinate intercept in figure 1 represents only 25% of total ^3H associated with the cell. While a small component of the total uptake, it is nonetheless easily distinguished when uptake is measured over this interval. If, however, uptake were monitored from 1 to 10 min, a time frame commonly chosen for study, and linearity were sustained over this interval, then by 10 minutes, the ordinate intercept would represent only 4% of total ^3H in the cell. If these data were then graphed using 10 minutes and the total cell ^3H as the highest values on the abscissa and ordinate, respectively, this intercept could not be distinguished from an ordinate intercept directly through the point of origin. Thus, whether the uptake slope intercepts through the point of origin depends not only on whether there is an early rapid association of radiolabel with cells, but also on whether the uptake interval is short enough to ensure that this uptake component will be a sufficient percent of total uptake to be detectable. This is a critical consideration.

Rapid association of drug with the cell may represent not only surface absorption but a rapid uptake of drug into the intracellular compartment, quickly reaching a steady state followed by a slower, linear uptake phase (under conditions in which the drug concentration within the cell is constant) related to metabolism of the drug to radiolabeled products that do not penetrate the plasma membrane and consequently accumulate within the cell. According to the latter scheme, and as clarified by studies with FdUrd indicated below, when this rapid transport of the drug is energy-independent

[3]Abbreviations used are: FdURd, fluorodeoxyuridine; FdUMP, fluorodeoxyuridine monophosphate; FUra, fluorouracil; FUMP, fluorouridine monophosphate.

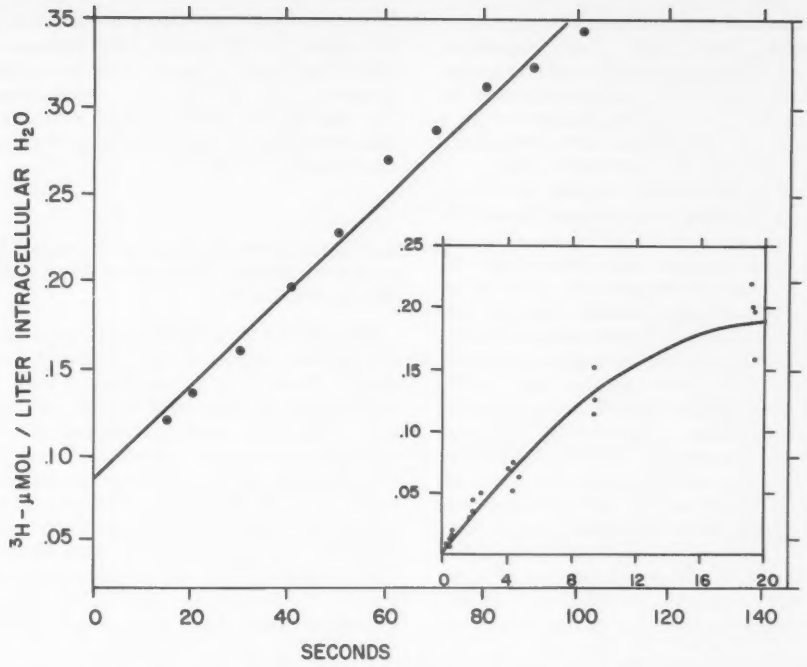

FIGURE 1.—Time-course of uptake of ^{3}H 20 to 100 sec after exposure of Ehrlich ascites tumor cells to 0.2 μM [^{3}H]-FdUrd. Uptake of ^{3}H was halted by injection of 10 vol of 0° [^{3}H]-FdUrd-free buffer containing sufficient dipyridamole to block influx. Extracellular material was removed by two washes with this solution. The inset is a separate experiment with rapid sampling (1) that amplifies uptake of ^{3}H over the first 20 sec and demonstrates the initial uptake phase.

(equilibrating[4]), the ordinate intercept should reflect a drug level within the range of the extracellular concentration. This analysis requires (*a*) that drug associated with the cell is expressed in units that can be compared to the extracellular drug concentration, ie., cell radiolabel per volume of intracellular water, *as if* the drug were osmotically active within the intracellular compartment, and (*b*) that the interval over which uptake is measured be brief enough to permit discrimination of an ordinate intercept comparable to, or some reasonable fraction of, the extracellular level.

A high ratio of apparent intracellular to extracellular drug concentration based upon an analysis of the ordinate intercept raises the suspicion that this may represent drug within the intracellular compartment rather than a surface adsorption. Often, an analysis of the uptake slope and ordinate intercept at 0° is employed to distinguish between surface adsorption and transport. The assumption is that transport is temperature-sensitive and will be slowed but that surface adsorption, an exothermic process, will not be altered. However, not all mediated transport processes are highly temperature sensitive; while passive diffusion of some substances can be modestly temperature sensitive (particularly hydrophilic compounds), most are not (9). Furthermore, when transport is very rapid at 37°, even a many-fold decline in uptake rate at 0° may not prevent a steady state from being achieved by the earliest time that intracellular radiolabel can be measured. Surface binding can be differentiated from rapid transport into the cell by identifying metabolites of the agent in the cell within an interval over which total uptake is essentially the same as the ordinate intercept (ie, < 5 seconds in fig 1). This indicates that some drug must have penetrated into intracellular compartment over this interval.

Another property of the ordinate intercept that raises the possibility of rapid transport rather than surface adsorpotion is the demonstration that this uptake component can be perturbed by inhibitors. For in-

[4]The term "equilibrating" is used to described a transport system that does not sustain a transmembrane electrical-potential difference for the transported species at the steady state.

stance, depression of the ordinate intercept by a metabolic poison would imply energy-dependent transport since a surface adsorption would be energy independent. Depression of the ordinate intercept by an analog raises the possibility of competition for a transport carrier, but competition for a low-affinity surface binding site must be considered as well.

Based on these considerations, a number of observations can be made regarding the rapid association of ^3H with cells after exposure to [^3H]-FdUrd: the ordinate intercept in figure 1 indicates that intracellular ^3H, expressed as per liter of intracellular water, is an appreciable fraction of (approximately one half) the extracellular drug level; dipyridamole depresses the ordinate intercept to near the point of origin and markedly inhibits the subsequent uptake slope while thymidine and deoxyuridine depress the uptake slope with a much lesser change in the ordinate intercept (fig 2); low levels of intracellular FdUMP and FUra can be detected within the cell 0.6 seconds after exposure to [3H]-FdUrd (1); finally, as described further below, FUra appears within the extracellular compartment more rapidly than the rate of accumulation of intracellular ^3H over the 20–100-seconds uptake interval. These observations suggested an initial rapid uptake of ^3H into these cells followed by a decline to a slower velocity within 20 sec. This was verified by analysis of ^3H uptake 0.5–20 seconds after exposure of cells to [^3H]-FdUrd (inset to figure 1). An initial linear uptake phase is now defined that extrapolates virtually through the point of origin. After 4 seconds, there is a decline in the net ^3H uptake velocity that falls to a constant rate by 20 seconds.

Correlating the Kinetics of Cellular Uptake of Radiolabeled Tracer with Drug Metabolism Within the Cell

The kinetics of ^3H uptake after exposure of cells to [^3H]-FdUrd suggested the following sequence of events. There is an initial linear uptake phase that represents a unidirectional flux of FdUrd into these cells. Over this interval, backflux of radiolabel is negligible, and uptake reflects membrane transport of FdUrd alone uninfluenced by subsequent intracellular events. As increased levels of free FdUrd accumulate within the intracellular compartment, backflux of FdUrd increases resulting in a rapid decline in the net FdUrd uptake rate so that by 20 seconds, the concentration of unchanged [^3H]-FdUrd within the intracellular compartment is at steady state. Following

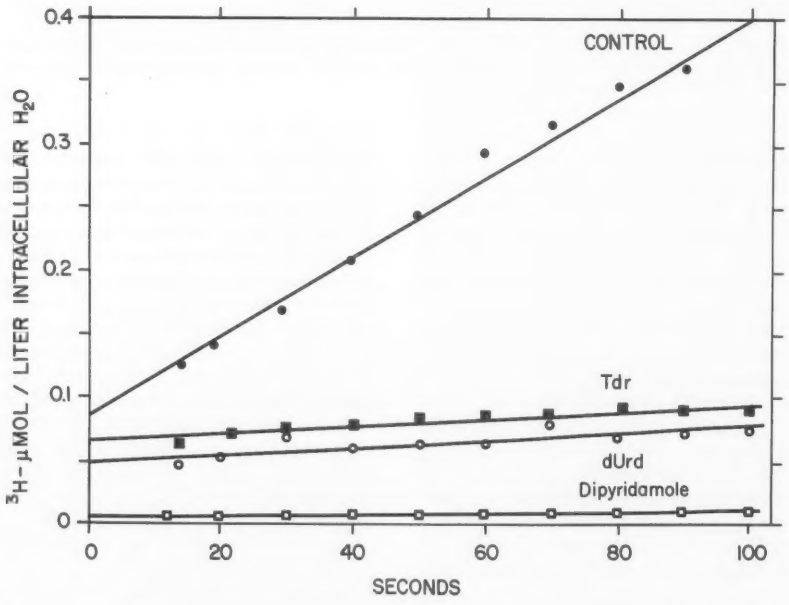

FIGURE 2.—Time-course of uptake of ^3H after exposure of cells to 0.2 μM [^3H]-FdUrd in the presence or absence of 10 μM thymidine (TdR), 100 μM deoxyuridine (dUrd), or 100 μM dipyridamole. Cells were processed as described in figure 1 (1).

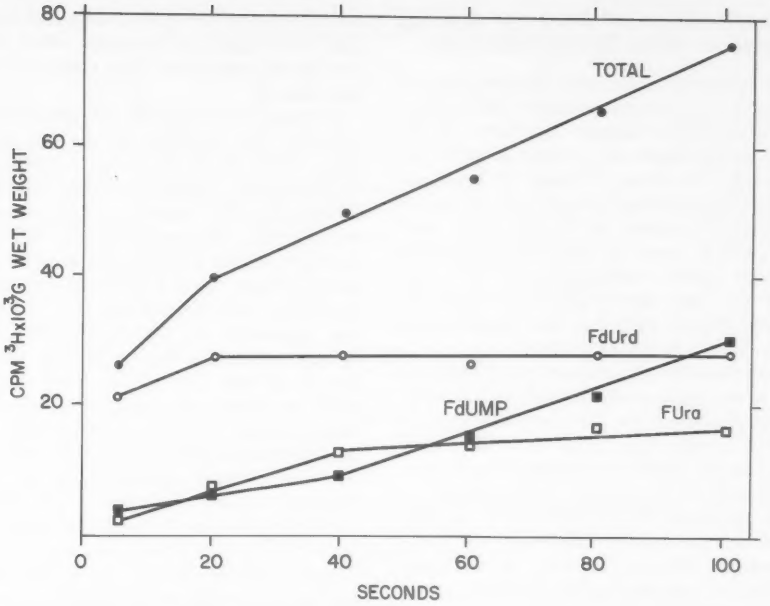

FIGURE 3.—Composition of intracellular ^{3}H was analyzed by high performance liquid chromatography after exposure of cells to 0.2 μM [^{3}H]FdUrd. Uptake was stopped by the addition of 10 vol of 0° buffer containing dipyridamole. After centrifugation, the cell pellet was dispersed in 0.5 ml of the 0° dipyridamole solution, 0.5 ml of 10% trichloroacetic acid was added, and the supernate was further prepared for high performance liquid chromatography as described in detail in ref 1.

this, continued net uptake of ^{3}H within the intracellular compartment represents metabolism of FdUrd to phosphorylated derivatives that do not penetrate the cell membrane.

It can be established from experiments such as figure 1 that the true initial uptake velocity for FdUrd is, in fact, at least six times faster than the uptake of 3H during the subsequent linear phase. This evaluation of ^{3}H uptake over the first 100 seconds after exposure of cells to [^{3}H]-FdUrd is confirmed by high performance liquid chromatographic (HPLC) analysis of the composition of intracellular ^{3}H over this interval (fig 3). By 20 seconds, the level of FdUrd within the intracellular compartment is at steady state and uptake of total cell 3H from 20–100 seconds parallels the rate of accumulation of FdUMP.

HPLC analysis contributes another important element that is not readily predictable from the time-course of uptake of ^{3}H alone. It demonstrates rapid hydrolysis of FdUrd to FUra. Intracellular FUra appears within only 0.6 seconds (1) and continues to accumulate over the uptake interval. The extent to which FUra accumulates in the cells depends not only on the rate of hydrolysis of FdUrd relative to the rate of FdUMP and FUMP synthesis and the rate at which FUra leaves the intracellular compartment, but on the size of the extracellular compartment as well. The latter determines the rate at which the extracellular FUra concentration increases, and this determines the rate of net loss of FUra from the intracellular compartment. Furthermore, the larger the extracellular compartment, the longer it will take to deplete extracellular FdUrd. As indicated above, the rapid appearance of FUra within the extracellular compartment was another important factor in suggesting an initial rapid uptake phase for FdUrd. The rate of FUra accumulation in the extracellular compartment, as estimated from the extracellular FUra level 50 seconds after exposure of cells to FdUrd, was at least 3.5 times greater than the rate of accumulation of ^{3}H within cells over 20–100 seconds. This could only be accounted for on the basis of a more rapid flux of FdUrd into the cell prior to 20 seconds followed by metabolism to FUra and exit of FUra from the intracellular compartment. While this could also be explained on the basis of hydrolysis of FdUrd at the cell membrane, no evidence for the latter phenomenon could be detected in the Ehrlich ascites system and the cells were culture-negative for mycoplasma, an organism with phosphorylase activity.

Distinguishing Between the Effects of Analogs on Drug Transport versus Drug Metabolism

As indicated in figure 2, thymidine and deoxyuridine inhibit ^3H uptake after exposure of cells to [^3H]-FdUrd. The major effect is a marked reduction of the 20–100-second uptake slope with a lesser effect on the ordinate intercept. The latter is based on linear extrapolation of ^3H uptake when, in fact, there is a subtle curve to these points; uptake of ^3H is declining over this interval. HPLC analysis indicates that these agents markedly inhibit metabolism of FdUrd to FdUMP and FUra; there were no major changes in the steady-state cell FdUrd level, but the possibility of small effects could not be excluded (1).

While these nucleosides produce a small inhibition of FdUrd influx at the concentrations employed (1), since the system is equilibrating, they can only slow the rate at which FdUrd reaches equilibrium as they in turn simultaneously reach equilibrium across the cell membrane, unless the rate of metabolism is so rapid relative to influx that the intracellular FdUrd level is below equilibrium in the absence of inhibition by these nucleosides (see the following section). On the other hand, dipyridamole markedly reduces the initial uptake phase, indicating a profound effect on transport. To determine whether there is an additional effect on metabolism it would be necessary to analyze the composition of intracellular ^3H in the presence of this agent. Inhibition of influx to this extent by dipyridamole should make transport rate limiting to metabolism so that virtually all intracellular ^3H should be metabolized unless the metabolic site is also inhibited.

Analysis of the Steady-State Level of Intracellular Drug Achieved

A variety of factors determine the steady-state level of FdUrd achieved within the intracellular water. First, there is no evidence that the transport system is itself energy-dependent, that it can produce sustained uphill transport of FdUrd into or out of the intracellular compartment (1), hence, this is a symmetrical carrier transport system (carrier kinetics are confirmed by the demonstration of trans-stimulation and counter transport (1,9). However, whether the transport system can actually achieve equilibrium for intracellular FdUrd depends on the rate of transport of FdUrd into the cell relative to the rates of phosphorylation to FdUMP and hydrolysis to FUra. The more rapid the metabolic steps relative to the rate at which the compound can be delivered into the cell, the lower the steady-state level achieved and the farther this value will deviate from equilibrium. Therefore, a steady-state transmembrane gradient for FdUrd could be sustained at an intracellular level lower than the extracellular concentration, not because of an active efflux mechanism but because of rapid metabolism relative to the rate of flux into the cell.

Likewise, these factors will determine the ordinate intercept. For instance, as the rate of FdUMP synthesis is increased, the ordinate intercept is decreased and the steady-state intracellular FdUrd level declines. Ultimately, this reaches a limit when the rate of metabolism is so rapid that free FdUrd does not accumulate within the intracellular water, transport (the unidirectional flux of FdUrd into the cell) becomes rate-limiting to metabolism, and the 20–100-second slope has increased to the extent that it equals the 0–4-second uptake slope. The zero time ordinate intercept is then through the point of origin and any point measured over the linear uptake phase is an accurate assessment of the unidirectional flux of FdUrd into the intracellular compartment, even though all ^3H in the cell is metabolite. It should be pointed out that transport and metabolism may not have a fixed relationship under physiological conditions, but may vary with the metabolic activity of the cell. Under some conditions, metabolism may be slower than transport; under other conditions, the metabolic rate can increase and ultimately make transport rate-limiting. For instance, Urd transport in 3T3 cells becomes rate-limiting to subsequent metabolism only when the rate of Urd phosphorylation is increased by serum activation (6).

From this consideration it follows that the identification of an unchanged drug, in this case FdUrd, within the intracellular compartment is an important clue to the relative rates of metabolism vs transport. When transport is rate-limiting to metabolism, unchanged drug cannot be detected within the cell; but when transport is rapid with respect to metabolism, unchanged drug can be identified within the intracellular compartment to the extent to which these velocities are different. Ultimately, of course, analysis of transmembrane gradients for a drug requires discrimination between drug that is free within the intracellular water and drug bound within or onto the cell. This requires analysis of drug efflux kinetics.

Compartmental Analysis of Radiotracer Efflux Kinetics: An Approach to the Assessment of the Transmembrane Gradient for Drug and the Rate of Drug Metabolism

Studies with [^3H]-FdUrd

Figure 4 illustrates an experiment in which cells were exposed to 0.2 μM [^3H]-FdUrd for 25 or 100 seconds, after which the cells were washed at 0° then resuspended into a larger volume of FdUrd-free medium at 37°.

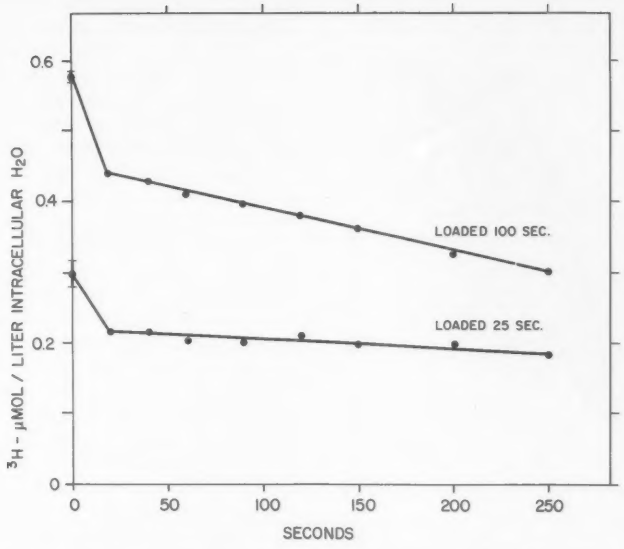

FIGURE 4.—Exchangeability of intracellular ^{3}H after incubation of cells for 25 or 100 sec with 0.2 µM [^{3}H]FdUrd. The cells were washed in 0° buffer after the initial incubation then centrifuged and resuspended into a large volume of [^{3}H]-FdUrd-free buffer at 37° (1).

It can be seen that by 25 seconds, there is about 0.1 µM rapidly exchanging ^{3}H within the intracellular compartment. However, there also is a large, slowly exchanging fraction that increases in size considerably as the duration of initial incubation is increased to 100 seconds. It can be established by HPLC that the rapidly exchanging component is largely FdUrd with a lesser amount of FUra while the slowly exchanging component is FdUMP. If uptake of ^{3}H is stopped sufficiently early after exposure of cells to [^{3}H]-FdUMP, the rapidly exchanging component would approximate the total ^{3}H level in the cell, and HPLC analysis would indicate that the major intracellular ^{3}H component is FdUrd (1). Thus this approach quantitates the rapidly exchanging component, one important step in analysis of potential transmembrane gradients, and excludes the contribution of high-affinity drug binding (within the cell or on the cell surface) from what is considered to be the osmotically active drug pool (loose binding may not be detected by this approach).

Documentating the accumulation rate of the slowly exchanging intracellular component can be useful in estimating the rate of metabolism of an agent to derivatives that do not, or only slowly, penetrate the cell membrane. For example, extrapolating the slopes describing the loss of the slowly exchanging ^{3}H component back to zero time in figure 4 quantitates this compartment size at the two times it was measured. The rate of increase of this compartment size equals the net rate of accumulation of total ^{3}H over 20–100 seconds and is equivalent to the rate of increase of intracellular FdUMP. Finally, the rate of loss of the slowly exchanging component either reflects the finite loss of metabolite from the cell or its conversion within the cell to other derivatives of the parent compound that, in turn, penetrate the cell membrane more rapidly. The latter possibilities are easily evaluated by analyzing radiolabel in the intracellular and extracellular compartments after efflux.

Studies With [^{3}H]-Deoxyuridine

Figure 5 illustrates a detailed analysis ^{3}H efflux after incubation of Ehrlich ascites tumor cells with [^{3}H]-deoxyuridine, a study undertaken to evaluate [^{3}H]-deoxyuridine cellular pharmacokinetics as a prelude to its use as an indicator of thymidylate synthesis and thymidine incorporation into DNA (2). Net accumulation of ^{3}H is indicated by the rising solid line through the open points. Extrapolation of the line to zero time demonstrated an ordinate intercept above the extracellular deoxyuridine level. At the arrows, cells were washed and resuspended into [^{3}H]-deoxyuridine-free buffer and the falling solid line represents rapidly exchanging and slowly exchanging ^{3}H components. The horizontal interrupted line extrapolates back to the

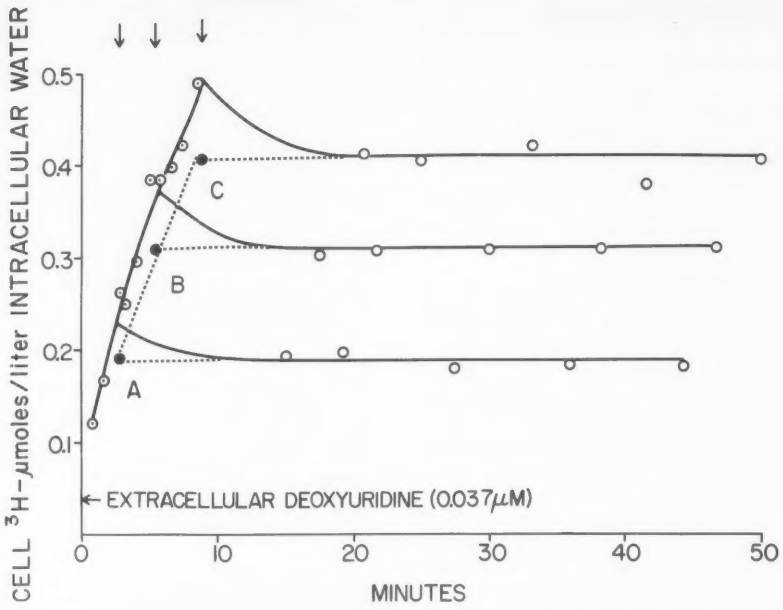

FIGURE 5.—Time course of uptake and efflux of ^{3}H after exposure of Ehrlich ascites tumor cells to 0.037 μM[^{3}H]-deoxyuridine. The rising continuous line is the time-course of ^{3}H uptake. Intracellular ^{3}H was determined after the cell fraction was washed twice with 0° buffer. At the arrows, portions of the cell suspension were separated by centrifugation, washed in 0° buffer, then resuspended into [^{3}H]-deoxyuridine-free buffer at 37°. The time-course of efflux is indicated by the descending continuous line. The horizontal interrupted line extrapolates back to the solid point and is the slowly exchanging component at the time when the cells were separated from [^{3}H]-FdUrd. The rising interrupted line connects these points indicating the rate of accumulation of slowly exchanging ^{3}H (2).

closed points to indicate the slowly exchanging level within the cell at the time the cells were separated from [^{3}H]-deoxyuridine. The rising interrupted line through these points therefore indicates the rate of accumulation of slowly exchanging ^{3}H. It can be seen that the slowly exchanging component can account almost entirely for the net accumulation of ^{3}H within the cells and must represent synthesis and retention of phosphorylated derivatives of ^{3}H deoxyuridine (incorporation into DNA is a negligible component of total cell ^{3}H over the first 5 to 10 min of uptake) (2). The exchangeable ^{3}H level (the difference between total and slowly exchanging ^{3}H at any time) is nearly constant. These data suggest a rapid initial uptake phase for deoxyuridine reflecting influx, and rapid achievement of a steady state for deoxyuridine within the intracellular water followed by slower metabolism to derivatives that do not penetrate the cell membrane by the time the first uptake point could be measured (2).

The Washing Technique—Stopping the Uptake Reaction and Washing Away Extracellular Drug and Metabolites

The technique employed to exclude radiolabeled drug and metabolites associated with the extracellular space of a cell pellet is critical in quantitating intracellular composition. The assumption is often made that washing at a low temperature removes extracellular but not intracellular material. This is not, in fact, the case for FdUrd. The transport system is not sufficiently temperature sensitive to prevent loss of intracellular material even with a 0° wash. Hence, by this technique, the cell FdUrd level is underestimated. Recent studies indicate that the wash removes even more FUra, so that intracellular base content is subject to even greater underestimation.

When the wash removes intracellular material, other techniques for excluding extracellular radiolabel from

total radiolabel associated with a cell pellet are required. A variety of approaches are possible including the use of an extracellular marker such as inulin (to quantitate the size of the extracellular space) with transport inhibitors (to block transport instantaneously) followed by isolation of the cell pellet by centrifugation (9,10). While this may result in rapid cessation of transport (the possibility that the inhibitor may produce a countertransport of substrate from the cell as well as block influx must be excluded), metabolism may continue during centrifugation and processing. Alternatively, high-speed centrifugation through silicone following rapid mixing of cells with substrate permits early uptake measurements, minimizes the extracellular space, and achieves rapid cessation of metabolic events when the cells are centrifuged through silicone directly into an acid compartment (7,8). However, when transport achieves a steady state in less than a few seconds this technique may not be rapid enough to document the initial uptake phase or early metabolic events. Indeed, no conventional technique is available to evaluate transport of minute quantities of substrate when the flux interval required is less than 0.5 second, and it should be emphasized that for many compounds, the documentation of initial rates may require measurements much earlier than 0.5 seconds.

CELLULAR PHARMACOKINETICS OF ACTINOMYCIN D

Just as differences in the rates of transport and metabolism influence the uptake sequence of a drug that is metabolically transformed within the cell, these considerations are relevant, as well, to agents that are bound within the cell. The cellular pharmacokinetics of actinomycin D, for instance, are very complex (3). The time-course of uptake of this agent into the Ehrlich ascites tumor is illustrated in figure 6 (the drug is not metabolized by these cells over this interval) (3).

There is an initial phase confirmed to be linear to an earliest time point of 20 seconds, with an ordinate intercept above the point of origin. On review of many such experiments, the ordinate intercept is variable and may be less than an order of magnitude below the extracellular concentration. Between 4 and 12 minutes after addition of actinomycin D, there is a decline in the uptake rate to a lower constant velocity that cannot be attributed to a fall in the extracellular actinomycin D concentration (it is essentially constant over this interval). Since the decline in the uptake slope is subtle and the binding capacity for these cells immense (see below), it can be seen that if uptake were measured over a much longer interval and graphed accordingly, the uptake points would tend to linearize and the curvilinear nature of the process would not be detectable.

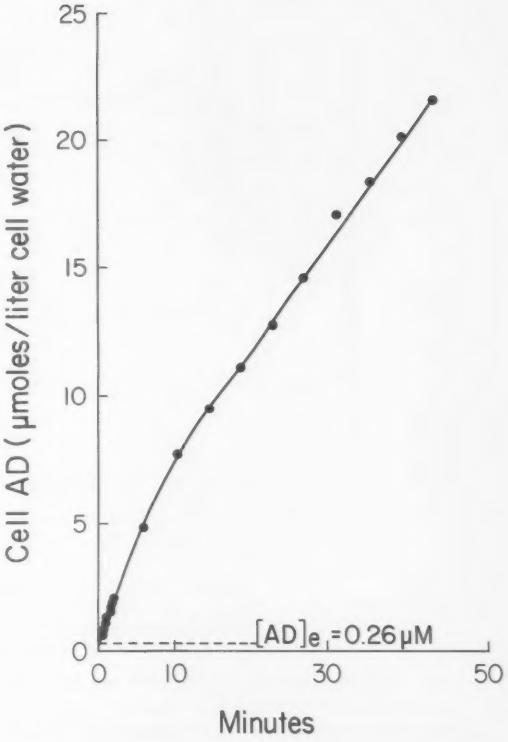

FIGURE 6.—Time-course of uptake of actinomycin D (AD) after exposure of Ehrlich ascites tumor cells to 0.26 μM tritiated drug. Intracellular radiolabel was confirmed to be AD by silica gel chromatography (3). The cells were washed in 10 vol of 0° buffer for determination of intracellular ^3H (3).

Likewise, a small ordinate intercept would become imperceptible.

Actinomycin D efflux characteristics, illustrated in figure 7, indicate that rapidly exchanging drug is present within the cell by 3.3 minutes after exposure to actinomycin D, and beyond this, a slowly exchanging component increases at a rate that approximates the net uptake of total actinomycin D over this interval. The basis for the two linear uptake components is unclear. It was initially assumed that the early linear portion reflects influx, rate-limiting to intracellular tight binding; the subsequent decline in rate reflects a rise in free intracellular actinomycin D to the steady state followed by a slower rate of binding. However, free drug is present in the cell before the initial linear component declines, and there is a significant, albeit

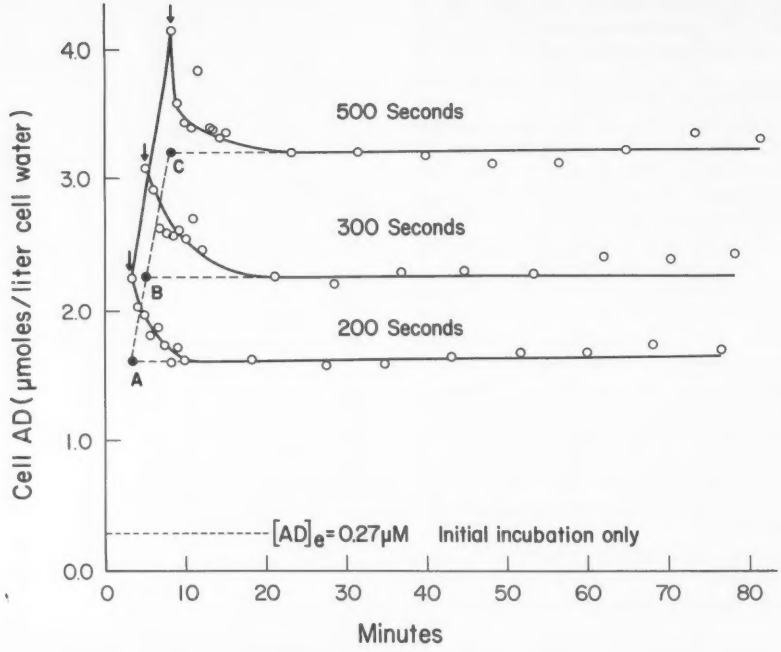

FIGURE 7.—Analysis of the exchangeability of actinomycin D (AD) after cells were loaded with drug for 200, 300, or 500 sec. At the arrows, exchangeability was determined as described in figure 5 (3).

variable, ordinate intercept. This raises the possibility of departure from initial uptake rates *before* the first measured linear phase and that the two observed linear uptake components may represent binding to different sites, one of low capacity but high affinity, saturated early in the uptake sequence, followed by binding to one or more sites with higher capacity but lower affinity. Further evaluation of the time course of actinomycin D uptake and exchangeability therefore is required to exclude the possibility of a rapid uptake component achieving steady state for free intracellular actinomycin D prior to 20 seconds. Be this as it may, the demonstration that exchangeable" intracellular actinomycin D accumulates, some of which must represent free drug, at the same time that binding continues indicates that transport must be faster than binding at some phase(s) of the uptake sequence. Alternatively, this could be related at least in part to slow transport into an intracellular compartment in which the drug is subsequently bound. The observation from figure 7 that rapidly exchanging drug is at a much higher level than extracellular actinomycin D suggests that this component is composed of both free and loosely bound actinomycin D within the cell. There is no evidence for energy-dependent transport into the cell that might offer an alternative basis for this apparent gradient.

Compartmental analysis of efflux kinetics not only permits the detection of binding per se, or sequestration of metabolites as in the case of FdUrd (see section on Compartmental Analysis, above), it permits the discrimination of multiple binding sites if their binding constants are sufficiently different. For instance, figure 8 illustrates the time-course of dissociation of actinomycin D from cells after loading for 3 or 40 minutes. The rapid efflux component can only be detected after a brief loading interval because it is too small a component of total actinomycin D to be detected when uptake is continued over the longer interval. Analysis of drug exchangeability therefore should be undertaken with a loading interval short enough to allow an intracellular level comparable to the extracellular concentration to be distinguished from total drug. After 40 minutes, two tightly bound fractions are detected. Neither are as tightly bound as the slowly exchanging component detected after loading for 3 minutes, indicating at least three different binding sites. Detection of multiple binding sites therefore requires analysis of efflux at different intracellular drug levels.

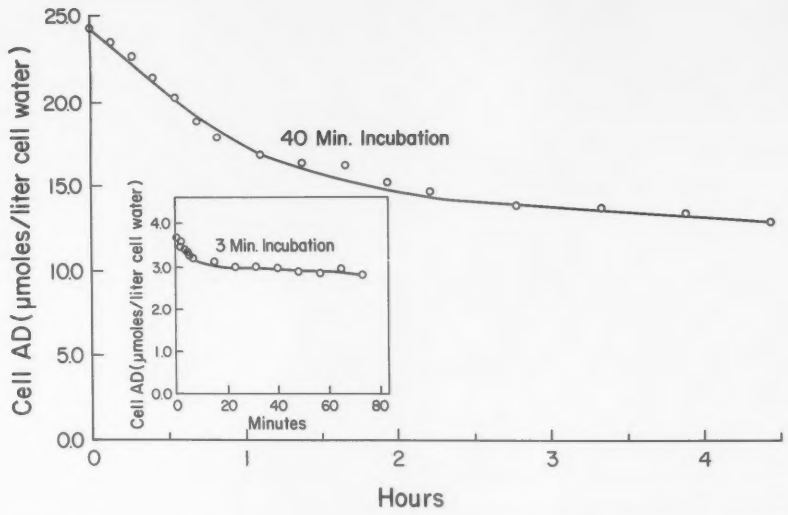

FIGURE 8.—A comparison of the exchangeability of intracellular actinomycin D (AD) after loading with 0.26 μM drug for 3 (inset) or 40 min. Cells were washed in 0° AD-free buffer prior to resuspension into a large volume of drug-free medium at 37° (2).

In summary, studies on cellular pharmacokinetics of anthracycline provide an approach for distinguishing between membrane transport and intracellular binding. From the time-course of uptake and unidirectional efflux of drug in the Ehrlich ascites tumor, multiple binding sites with different affinities for this agent can be identified. However, distinguishing between transport and binding-determined aspects of the uptake sequence, based on the recent awareness of the need for evaluation of rapid uptake components, will require further investigation. The magnitude of the cell capacity to bind actinomycin D and other anthracyclines relative to the quantity in the cell that represents free drug at equilibrium with extracellular drug or other small bound components is so great that if the uptake interval is sufficiently long, these compartments, as well as the initial ordinate intercept, may not be detected at all. This could lead to the erroneous conclusion that transport is rate-limiting to all intracellular binding. Clearly, these considerations are of considerable importance in establishing (*a*) rate limiting steps in the association of anthracyclines with cells, (*b*) mechanisms of resistance, and (*c*) correlations between bound anthracycline components within the cell and the pharmacologic and cytotoxic effects of these agents.

CELLULAR PHARMACOKINETICS OF METHOTREXATE WITH PARTICULAR REFERENCE TO THE SYNTHESIS OF METHOTREXATE POLYGLUTAMATES AND BINDING TO SITES OTHER THAN DIHYDROFOLATE REDUCTASE IN FRESHLY ISOLATED RAT HEPATOCYTES

Cellular pharmacokinetics of methotrexate have been studied extensively in a variety of mammalian cell systems. Many aspects of the membrane transport of this agent have been described, although this complex process is not yet completely understood (11,12). Possibly more important, however, is the contribution that this experimental approach has provided to understanding the interaction within the intracellular compartment among the drug, its target enzyme dihydrofolate reductase, and the substrate dihydrofolate and the role the membrane transport system plays in this phenomenon (13–16). This has led to the recognition that methotrexate is a very rapidly reversible inhibitor of dihydrofolate reductase activity within the cell due to (*a*) the low level of enzyme activity required to sustain cellular tetrahydrofolate synthesis, and (*b*) the high levels of dihydrofolate substrate that accumulate within the cell as methotrexate associates with the

bulk of enzyme resulting in (c) potent competition between dihydrofolate and methotrexate for the small but critical percentage of total enzyme sites (13–16).

This perspective clearly indicated that the rate of flux of methotrexate into the cells in terms of "stoichiometric inactivation" of dihydrofolate reductase is not the critical factor in drug transport. Rather, it is the level of free drug achieved and sustained within the intracellular compartment (9,13,15–18) that are the important elements controlled by the membrane transport system. These parameters of the drug-cell interaction parallel drug influx (9,19) and often can be related to the affinity of the carrier for methotrexate at the outer cell membrane (19). The basis for this understanding came from a highly quantitative analysis of methotrexate interaction with cells, a clear definition of bound and non-bound fractions, and the role of these components in methotrexate inhibition of macromolecular and tetrahydrofolate synthesis within the cell.

More recently, this experimental approach has been directed to evaluation of other elements in methotrexate pharmacology, metabolism to polyglutamate derivatives (4), and binding to sites other than dihydrofolate reductase (5). The synthesis of methotrexate polyglutamates (20–23) may have important pharmacologic implications. Methotrexate is a rapidly reversible inhibitor of dihydrofolate reductase activity within the cell because it rapidly leaves the cell as the extracellular level falls. Polyglutamates of methotrexate retain a high affinity for dihydrofolate reductase (24). If they do not readily penetrate the cell membrane, this would convert the drug to a very slowly reversible or irreversible inhibitor of the enzyme as high intracellular levels of pharmacologically active derivatives are sustained while the methotrexate blood level falls (25). Differences in the synthesis of methotrexate polyglutamates between tumor cells of differing sensitivity to this agent or tumor versus susceptible host tissues could play an important role in selectivity, cytotoxicity, and resistance and is an area that requires further exploration.

If, as observed for phosphorylated derivatives of FdUrd, the polyglutamate metabolites of [^{3}H]-methotrexate are retained within the cell, this should produce predictable alterations in the time-course of uptake and exchangeability of intracellular ^{3}H after exposure of cells to [^{3}H]-methotrexate. In many systems that do not form appreciable levels of methotrexate polyglutamates over short intervals, such as the L1210 leukemia or the Ehrlich ascites tumor, uptake of drug reaches steady state within 30 minutes. If synthesis of methotrexate polyglutamates occurs at an appreciable velocity so that an increase in net accumulation of ^{3}H on this basis can be detected from the larger level of total drug within the intracellular compartment, a slow linear uptake of ^{3}H will be observed instead of the steady-state plateau. Likewise, studies on exchangeability of intracellular ^{3}H at various times after exposure to [^{3}H]-methotrexate will show a comparable continued increase in a slowly exchanging ^{3}H fraction after methotrexate is present in the cell in an amount in excess of the dihydrofolate binding capacity.

Recently, studies using the freshly isolated rat hepatocyte as well as hepatocytes in monolayer culture have revealed not only rapid synthesis of methotrexate polyglutamates (4,20) but the binding of methotrexate to high-affinity site(s) in addition to dihydrofolate reductase (5). Figure 9 is an analysis of the time-course of association of ^{3}H with freshly isolated rat hepatocytes exposed to 1 μM [^{3}H]-methotrexate. Uptake is multiphasic with an increasing accumulation of slowly exchanging ^{3}H to levels far in excess of the dihydrofolate reductase binding capacity.

Figure 10 is a quantitative analysis of rapidly and slowly exchanging methotrexate levels as well as cell methotrexate polyglutamates (determined by DEAE cellulose column chromatography) over 2–15 minutes (panel A, left) or 15–60 minutes (panel B, right) after exposure to [^{3}H]-methotrexate. Rapidly exchanging methotrexate accumulates prior to saturation of high-affinity binding sites other than dihydrofolate reductase, indicating that transport of methotrexate is not rate-limiting to binding to these intracellular sites. Alternatively, the time-dependent increase in slowly exchanging methotrexate could be based upon slow transport of drug into an organelle followed by tight binding. Rapidly exchanging methotrexate is at steady state within 30 minutes. Total uptake, however, continues due to accumulation of a slowly exchanging component that can be related only in part to the accumulation of methotrexate polyglutamates. The latter accumulate at only 60% the rate of accumulation of the total slowly exchanging component; and as the extracellular methotrexate level is increased, this difference increases as well. Methotrexate polyglutamates accumulate to an intracellular level three times greater than the extracellular methotrexate concentration and 60 times greater than the level of methotrexate polyglutamates that leave the cell and accumulate within the extracellular compartment over this interval (less than 5% of total extracellular ^{3}H, 0.05 μM (1)). This indicates that these methotrexate polyglutamates (largely MTX + G1 with a lesser amount of MTX + G2 (1)) are retained within the rat hepatocyte, either because they are bound or because they penetrate the cell membrane very slowly.

These cellular pharmacokinetic studies with methotrexate in the rat hepatocyte system offer the opportunity to characterize the kinetics and control of the synthesis of methotrexate polyglutamate derivatives in this cell system as well as the effects of these derivatives on the pharmacologic action of methotrexate.

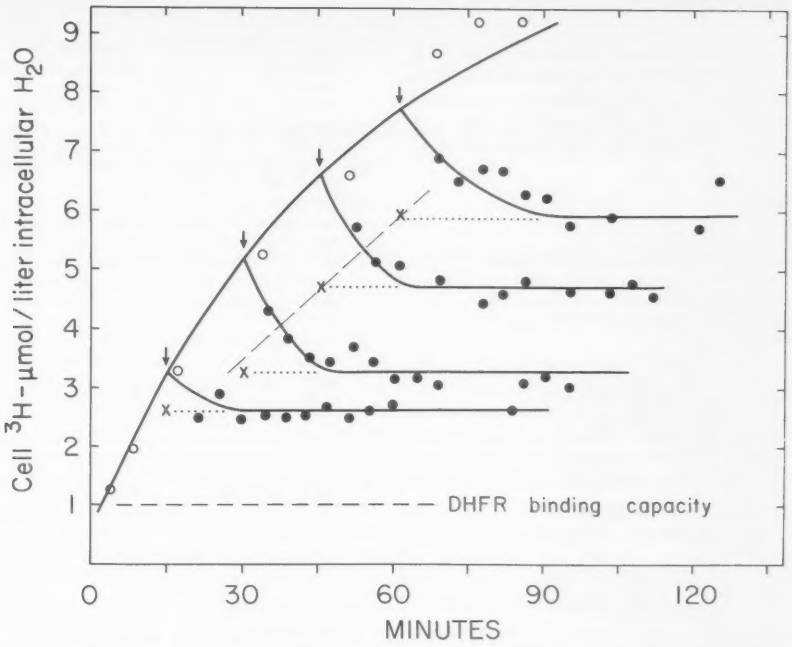

FIGURE 9.—Time-course of uptake and exchangeability of intracellular ^3H after exposure of freshly isolated rat hepatocytes to 1 µM [^3H]-methotrexate. At the arrows, exchangeability of intracellular ^3H was established by a 20-fold dilution of extracellular drug. The data indicate a time-dependent increase in a slowly exchanging intracellular fraction (rising interrupted line through the solid points) plotted as in figures 5 and 7.

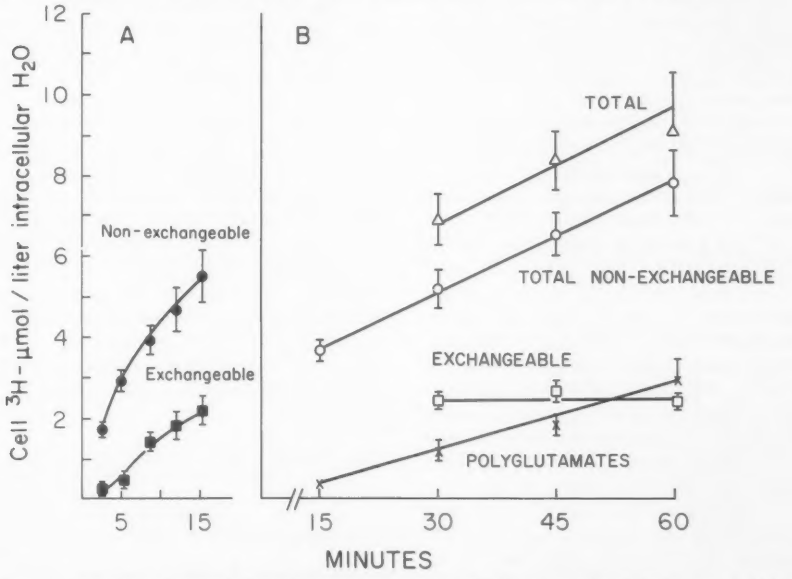

FIGURE 10.—Analysis of intracellular methotrexate components derived from several experiments, one of which is illustrated in figure 9, showing the rise of slowly and rapidly exchanging methotrexate and methotrexate polyglutamates in freshly isolated rat hepatocytes over the first 15 min (panel A, left) and 15–60 min after exposure to the drug (panel B, right).

Identification of intracellular binding sites for methotrexate observed in this cell system will be of interest, especially the effect of this binding on the ability of methotrexate to inhibit dihydrofolate reductase and to serve as a substrate for polyglutamate synthesis.

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The Potential for Clinical Application of In Vitro Assays Predicting 5-FU Sensitivity in Man

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SUMMARY

An in vitro assay system to measure the production of phosphorylated nucleotide products of 5-FU by human tumor cytosols is described. Samples from 11 patients with breast cancer treated with 5-FU-containing chemotherapy regimens were assayed. Significant differences were seen in quantitative and qualitative patterns of phosphorylation between samples from chemotherapy responding and nonresponding patients. Both the rate of 5-fluorodeoxyuridylate production and the amount of fluorinated nucleotide triphosphate produced were significantly increased ($P<0.05$) in patients responding to chemotherapy. The latter finding may be attributed to the demonstration of significantly higher nucleoside diphosphate kinase levels in the tumors of responding patients. Further study of this in vitro assay system as a potential system for prospectively predicting clinical response to fluorinated pyrimidines is indicated.

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Heidelberger and colleagues (1,2) originally synthesized the fluorinated pyrimidine 5-FU, which potently inhibits the growth of a variety of tumor cells (3,4). Unfortunately, in clinical practice in patients with disseminated cancer of the ovary, bowel, and breast, 5-FU is associated with objective partial response in only 20%–30% of cases (5). An in vitro predictive assay to prospectively select patients possessing tumor responsive to 5-FU would be useful. Such an assay would result in avoiding the unsuccessful treatment of 70%–80% of patients with fluorinated pyrimidines and would allow selection of patients most likely to benefit from this class of drugs.

Fluorinated pyrimidine drugs must undergo metabolic activation to become effective antineoplastic agents (6). 5-FU, for example, must be extensively anabolized first to its nucleoside and then to nucleotide derivatives to demonstrate antineoplastic activity. Failure to perform these conversions may result in resistance to this drug (7). Because of the complicated activation of this agent, resistance can emerge in one or several steps of its anabolism to active nucleotides.

We have reported (7) on an assay system that predicted the response of murine tumors to fluorinated pyrimidines. In this assay, the ability of cell-free supernatants of tumors to produce phosphorylated products of 5-FU correlated with in vitro sensitivity of the tumors to 5-FU. Murine tumors sensitive or resistant to 5-FU in vivo demonstrated distinctly different in vitro 5-FU phosphorylation patterns. For example, when the pattern of phosphorylation in the 5-FU-sensitive tumor P388S was examined, it was seen that nucleoside mono-, di-, and triphosphates were formed. This was in contrast with the resistant tumor P388R in which only monophosphates were detected. There was no significant difference in the concentration of the monophosphate peak in the sensitive or the resistant lines, whereas differences in the levels of di- and triphosphate peaks were statistically significant. 5-Fluorodeoxyuridylate (FdUMP) generation was minimal in the murine tumors tested.

This report discusses the application of our assay system to human breast cancer specimens. We have correlated the ability of extracts of human tumors to form phosphorylated products of 5-FU with clinical response to chemotherapy.

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MATERIALS AND METHODS

Patients

Eleven patients with metastatic or locally advanced primary breast cancer were studied. Biopsy specimens were trimmed of excess fat and portions of tumors for 5-FU anabolism assays were frozen within 15 minutes in liquid nitrogen. The ability of cell-free tumor extracts to produce phosphorylated products of 5-FU was assayed on prechemotherapy specimens of all patients. The patients were treated with 5-FU-containing combination chemotherapy regimens (cyclophosphamide, methotrexate, and 5-FU or cyclophosphamide, adriamycin, and 5-FU).

In all patients, assessment of response was performed as follows. Complete response required the disappearance of all measurable disease including healing of all lytic bone lesions and a return of the patient to a premorbid performance status. Partial response required a shrinkage of 50% of all measurable disease. No new lesions could appear and no growth could be observed in a pre-existing lesion for at least 2 months. Any patient not achieving this degree of improvement was described as a nonresponder. All patient records were assessed by investigators unaware of 5-FU in vitro assay results.

Assay System

The basis of the assay system was the incubation of a cytosol fraction of tumor homogenate with ^{14}C-labeled 5-FU. Tumor homogenates were prepared from 50 mg of tumor with a hand homogenizer in 0.01 M Tris buffer containing 0.15 mM EDTA and 0.5 mM dithiothreitol (pH 7.4). The homogenates were centrifuged at 12,000 x *g*, and the cytosol fraction removed for the assay. The incubation medium consisted of the following substances: 10 μl of ^{14}C 5-FU (SA 9.1 mCi/mole) 10 μl of a buffer mix containing 0.03 M of the following sugars, ribose-1-phosphate (R-1-P), deoxyribose-1-phosphate (dR-1-P) and phosphoribosylpyrophosphate (PRPP). The reaction mixture also contained 0.03 M phosphoenolpyrophosphate, 10 units of pyruvate kinase and myokinase/ml of buffer mix, and ATP at 10 mM (pH 8.0). The reaction was started with the addition of 10 μl of cytosol fraction. To assess differences in the ability of the cytosols to utilize individual sugars as substrates, experiments were performed in which each cytosol was incubated in the above assay system with a single sugar, ie, R-1-P, dR-1-P, or PRPP.

The reaction mixture was incubated at 37 ° for 30 min since we had demonstrated that production of total phosphorylated products of 5-FU was linear over that time. The reaction was stopped by heating to 95 ° for 2 min followed by centrifugation at 12,000 x *g* for 1 min. The supernatants were then analyzed for phosphorylated products by the methods described below.

Total phosphorylation was assessed ultilizing paper chromatography. Five-μl aliquots of the acidified supernatant of the reaction mixture were spotted in duplicate on Whatman 3M paper. Ascending chromatography was performed overnight in a solvent system consisting of isopropanol, toluene, 0.1 M EDTA, and concentrated NH_4OH (320:40:44:4, V:V:V:V). The papers were air-dried and the UV-absorbent spots were circled, cut, eluted with 200 μl of 0.1 M HCl, and counted at 80% efficiency in a scintillation counter.

The mono-, di-, and triphosphates of 5-FU were alalyzed by high-resolution ion exchange chromatography. Reaction mixtures were scaled up 20 times, deproteinized, and centrifuged as previously described. One-ml aliquots of the clear supernatants were loaded on a 0.8 x 15 cm column of Hamilton HA- x 4 resin, thermoregulated at 37 °C. Elution of anions was accomplished at a flow rate of 0.67 ml/min with five stepwise buffers of lithium citrate at pH 2.65–2.72; then 0.015 M buffer for 90 minutes, followed by 0.0375 M, 0.075 M, 0.15 M, and 0.225 M lithium citrate buffers at the same pH, applied for periods of 60 minutes each. Under these conditions 5-FU and 5-fluoro-2-deoxyuridine (5-FUdR) eluted with the void volumes at 8 min, 5-fluorouridylate (FUMP) and FdUMP at 96 min, the diphosphates FUDP and FdUDP at 245 min, and the triphosphates FUTP and FdUTP at 330 min. Fractions were collected and the peaks of interest were desalted on miniature ammoniacal columns of acid-washed charcoal. Nucleotides were eluted from the column using 60% ethanol.

Determination of FdUMP generation was carried out by a modification of previously described (8) assays based on the inhibition of thymidylate synthetase by FdUMP. The major modification we made involves the microdistillation of the tritiated water resulting from methylation of 5-^{3}H-deoxyuridinemonophosphate (5-^{3}H dUMP). In analyzing the results of these assays, a low rate of anabolism of 5-FU by an individual breast tumor extract was arbitrarily defined as ≥ 1 but < 12 nmoles of phosphorylated products of 5-FU/mg protein/hr. A high rate of anabolism by a tumor cytosol was defined as ≥ 12 nmoles of phosphorylated products of 5-FU/mg protein/hr.

RESULTS

Eight of 11 patients were documented to have partial objective responses to 5-FU-containing combination chemotherapy regimens. There were no significant differences in performance status and site of metastases between responding and nonresponding patients. The ability of cytosol fractions from the tumors of these patients to produce phosphorylated products of 5-FU was assayed.

Synthesis of FUdR, FUR, and FUMP

Pyrimidine nucleoside phosphorylase catalyzes the reversible conversion of pyrimidine nucleosides to ribose-1-phosphate (R-1-P) or deoxyribose-1-phosphate (dR-1-P) pyrimidine bases. Also, FUMP may be formed directly from 5-FU via the enzyme phosphoribosyl transferase. To assess the activities of these enzymes in human breast cancer samples under study, [^{14}C] 5-FU was incubated with the cell-free supernatant in the presence of saturating concentration of R-1-P, dR-1-P and PRPP as described previously. There was a significant difference ($P < 0.05$) between the responders and nonresponders in the in vitro conversion rates of 5-FU to FUR but not to FUdR or FUMP (table 1).

Formation of Nucleotides from [2-^{14}C] 5-FU by Human Breast Cancer Cytosols

The subequent anabolism of the riboside or deoxyriboside of 5-FU involves phosphorylation of the sugars catalyzed by pyrimidine nucleoside kinases. The results of total phosphorylation for the assayed breast cancer samples are illustrated in figure 1. In the nonresponding patients, total phosphorylation of 5-FU usually proceeded at a rate that was slower than that seen in responders. However, one nonresponder, patient 11, generated 25 nmoles of 5-FU phosphorylated products. The differences in total phosphorylation between responding and nonresponding patients were not statistically significant.

To assess the total activities of the several pyrimidine nucleotide kinases required for di- and trinucleotide phosphate formation, the total phosphorylated products of 5-FU were resolved into mono-, di-, and triphosphates. Highly significant differences ($P < 0.0001$) between responders and

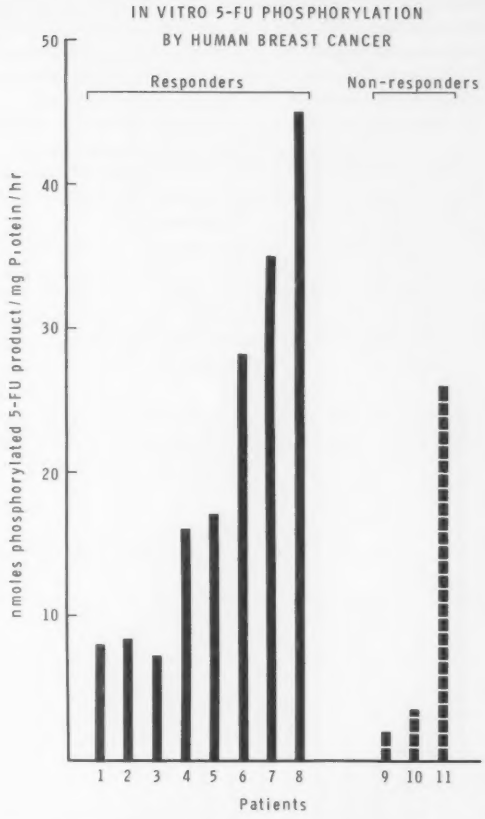

FIGURE 1.—Total production of phosphorylated nucleotide products of 5-FU by human breast cancer samples.

TABLE 1.—Synthesis of FUdR, FUR, and FUMP from 5-FU and cytosols from human breast cancer in vitro*

Precursors	Product	Responders†	Nonresponders†
dR-1-P	5-FUdR	307.0‡ ± 121.2	275.2 ± 125.1
R-1-P	5-FUR§	272.3 ± 98.2	81.5 ± 29.2
PRPP	5-FUMP	1.43 ± 0.59	1.67 ± 0.72

*Cytosol fraction of human breast cancer incubated with [2-^{14}C]5-FU and buffer mix containing dR-1-P, R-1-P, or PRPP

†nmoles of product formed/mg protein/hr

‡mean ± SD

§A significantly increased ($P < 0.05$) rate of FUR production in samples from patients responding to chemotherapy is demonstrated.

nonresponders were seen for rate of generation of triphosphates. All the responding patients' tumor samples were able to generate over 8 nmoles of FUTP/mg protein/hr; all the nonresponders generated less than 0.3 nmoles of FUTP/mg protein/hr (fig 2).

It was also possible to compare the activities of the mono- and diphosphate kinases in responding and nonresponding patients. This is illustrated in table 2, which compares the mean nmoles of diphosphate and triphosphate nucleotide produced/mg protein/hr. There was no significant difference of specific activity of nucleoside monophosphate kinase between responding and nonresponding patients. However, there was significantly increased ($P < 0.05$) diphosphate kinase activity in responding patients' tumor samples.

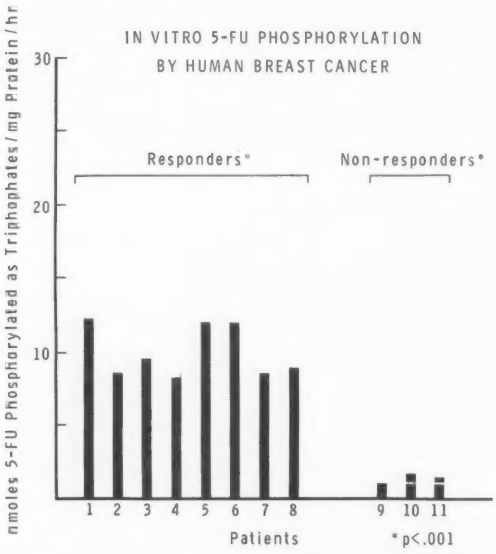

FIGURE 2.—Production of nucleotide triphosphates by human breast cancer samples. Samples from patients responding to chemotherapy produced significantly higher average amounts of triphosphates than nonresponding patients ($P < 0.001$).

FdUMP Generation by Human Breast Cancer Cytosols

All eight patients who clinically responded generated over 800 pmoles of FdUMP/mg proetin/hr compared with nonresponders who generated less than 200 pmol of FdUMP/mg protein/hr (fig 3). This result was significantly different ($P < 0.05$).

DISCUSSION

The preliminary results described in this communication have been developed from patients who were treated with combination chemotherapy and not single-agent 5-FU. However this results from the fact that it is inappropriate to treat advanced breast cancer patients with single-agent 5-FU because of the documented superiority of combination chemotherapy (9). The results in this assay were not affected by whether patients received CMF or CAF.

These results illustrate the applicability of our assay system to human tumor samples and also confirm that the differences in the ability of human tumors to anabolize 5-FU are analogous to those we have seen in the murine system (7). Significant differences are seen in the amount of triphosphate nucleotides of 5-FU formed. From examining the specific activities of nucleoside kinases (table 2), it is possible to determine that the increased production of triphosphates could be secondary to increased diphosphate kinase activity in the tumors of responding patients. Significant differences are also seen in the rate of generation of the potent thymidylate synthetase inhibitor, FdUMP, between responding and nonresponding patients. These data suggest that resistance to 5-FU in some human tumors may result from either the failure of the tumor to produce and sustain adequate amounts of FdUMP to inhibit DNA synthesis or the failure of production of sufficient amounts of FUTP to interfere with RNA synthesis and function.

It is possible that maximum cytotoxicity in some human tumors may result from a combination of the inhibition of DNA and RNA synthesis and function. This is supported by reported animal data suggesting the

TABLE 2.—Specific activity of "higher" kinases in cytosols from human breast cancer in vitro

	Responders	Nonrespnders
Nucleoside monophosphate kinase (nmoles/mg protein/hr)	4.8 ± 1.9* (8)†	2.7 ± 0.7 (3)
Nucleoside diphosphate kinase (nmoles/mg protein/hr)	9.5 ± 2.3‡ (8)	3.5 ± 1.7 (3)

*Mean ± S.D.
†Number of patients
‡$P < 0.05$

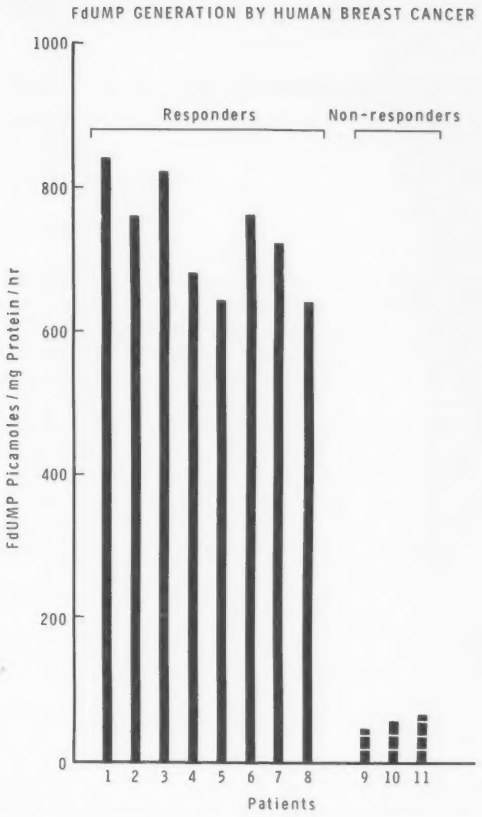

FIGURE 3.—FdUMP generation by human breast cancer samples. Samples from patients responding to chemotherapy had significantly ($P < 0.05$) increased FdUMP generation in comparison to samples from nonresponding patients.

importance of 5-FU inhibition of RNA as a mechanism of cytotoxicity. Sawyer and Martin and colleagues (10) have shown in the CD8F1 murine mammary tumor model that >90% inhibition of DNA synthesis only occurred at greater than 24 hr after 5-FU treatment. This finding was shown to correlate positively with the amount of fluorinated pyrimidine incorporated into RNA. The authors hypothesize that the mechanism of DNA synthesis inhibition appeared to be secondary to decreased levels of thymidine kinase resulting from 5-FU interfering with RNA translation and transcription.

The current studies reported here can only serve as the basis of suppositions concerning 5-FU activity in human tumors. However, the assay systems described, which are simple, reproducible, and can be performed on cryopreserved biopsy specimens, may be useful in helping to develop predictive assays of 5-FU activity in man. We plan a prospective study in which 5-FU anabolism will be studied in tumor cytosols from colon cancer patients. These patients will subsequently be treated with single-agent 5-FU, and response to chemotherapy will be correlated with in vitro 5-FU anabolism studies. This approach will convincingly test whether these in vitro assay systems can predict the clinical activity of 5-FU in man.

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The Target Cell Determinants of the Antitumor Actions of 5-FU: Does FU Incorporation Into RNA Play A Role?[1]

H. George Mandel[2,*]

SUMMARY

Sufficient evidence has accumulated to permit the conclusion that there are at least two major determinants for the effectiveness of 5-FU against tumors. These are the formation of fluorodeoxyuridylate (FdUMP) and the incorporation of 5-FU into RNA. Either or both of these determinants can be of critical significance in the tumor-inhibitory actions of 5-FU and the sensitivity of normal tissues to this drug, depending on the biological system and the experimental conditions. The critical role for FdUMP and inhibition of DNA synthesis as a cell target determinant is supported by the recognition of "thymineless death;" the time-course correlation of levels of this metabolite (and/or of susceptibility of thymidylate synthetase) with tumor sensitivity to 5-FU and with cytotoxicity to specific organs and tissues; and the reversibility of the actions of 5-Fu by thymidine in vitro. A role for 5-FU incorporated in RNA (FU-RNA) is based on various toxic consequences of the incorporation of the analog, including the inhibition of ribosomal maturation; the occasionally reported lack of rescue from growth inhibition with thymidine, which prevents the 5-FU induced inhibition of DNA synthesis, apparently with increaed FU-RNA formation; the activity of uridine as a partial rescue agent; and various studies revealing correlations of FU-RNA with enhanced therapeutic response.

[Cancer Treat Rep 65 (Suppl 3):63–71, 1981]

The uracil analog 5-FU is known to be metabolized to its 2'-deoxyribose phosphate derivative, fluorodeoxyuridine monophosphate (FdUMP) (1). This drug metabolite then forms a covalent complex with thymidylate synthetase and 5,10-methylenetetrahydrofolate, leading to the inhibition of thymidylate synthesis and of DNA formation (2,3). A second metabolic pathway, the conversion of 5-FU to a variety of ribonucleotides culminating in the formation of 5-FU incorporated into RNA (FU-RNA) (4), has also been recognized ever since the early investigations on this antitumor drug (fig 1). There has been considerable interest and controversy as to which of these two separate routes of transformation is primarily responsible for the drug's antitumor effectiveness. In almost all experimental systems it has been extremely difficult to discriminate between these two pathways and their consequent pharmacological effects since 5-FU is normally anabolized by both routes simultaneously (5).

TARGET CELL DETERMINANTS

It is generally assumed that the formation of FdUMP, leading to the inhibition of thymidylate synthesis, is the principal determinant of 5-FU sensitivity (6–8). Blockade of this enzyme and of DNA synthesis by FdUMP has been correlated with tumor growth inhibition in animal systems (9) and even in man (10), although these correlations were not always exact. The ability of exogenous thymidine to prevent the actions of 5-FU in certain tumor systems in vitro by supplying an alternative source of thymidylate supports the essential role of FdUMP in tumor growth inhibition.

Although it is recognized that the formation of FU-RNA can exhibit significant effects in specific biological systems, the importance of this abnormal RNA in inhibition of tumor growth or cytotoxicity has not been clearly demonstrated (reviewed in 8, 11). The analog has been shown to be incorporated into all classes of RNA, including ribosomal, transfer, and messenger RNA. In tumor cells 5-FU has been found

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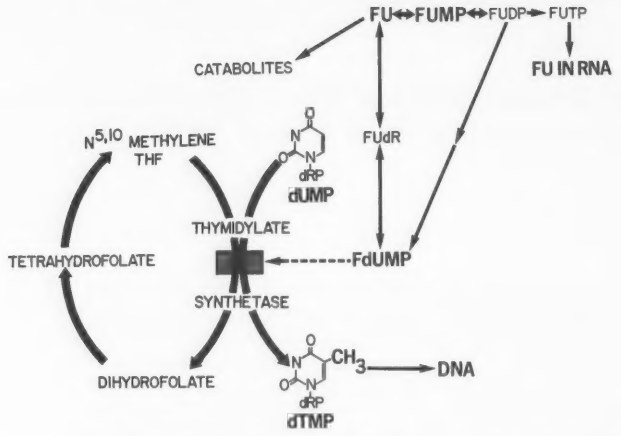

FIGURE 1.—Interrelationship of metabolism of 5-FU and pharmacological actions. FU is converted to FdUMP which inhibits thymidylate synthetase. This enzyme catalyzes the methylation of DUMP to dTMP. 5-FU is also incorporated into RNA after the formation of various FU-containing metabolite analogs. FdUMP = fluorouridine 5'-monophosphate; FUDP and FUTP = fluorouridine di- and triphosphate, respectively; FUdR = fluorodeoxyuridine; FdUMP = fluorodeoxyuridine 5'-monophosphate; dUMP, = deoxyuridine 5'-monophosphate; dTMP = thymidine 5'-monophosphate; and THF = tetrahydrofolate. From (5).

mainly in nuclear RNA. In microbial systems, there have been numerous reports of FU-RNA-produced physicochemical alterations of select enzymes, miscoding leading to changes in viral protein composition, inhibition of enzyme induction, and failure of ribosomal maturation. These last two effects also occur in mammalian systems (12-14).

It is the purpose of this report to review these two determinants of 5-FU action, with particular emphasis on the role of FU-RNA formation in the drug's antitumor actions. This topic has been the subject of renewed interest, stimulated in part by results in model tumor systems in which the effects of 5-FU on RNA synthesis might be the principal mechanism of antitumor action (15). Certain recent basic studies in which it was possible to dissociate the effects of 5-FU on DNA synthesis from those on RNA function have raised additional support for an important role of the latter in antitumor chemotherapy. In this report we will not consider other biochemical steps that might limit the formation of FdUMP or FU-RNA indirectly, such as those involving the synthesis of 5-fluorouridylate (FUMP) or 5-fluorodeoxyuridine (FUdR), the reduction of 5-fluorouridinediphosphate (FUDP), or the degradation of 5-FU and its derivatives.

Fluorodeoxyuridine Monophosphate

Considerable evidence has accumulated in support of the early hypothesis of Heidelberger et al (9) that the formation of FdUMP is crucial for the antitumor activity of 5-FU since most other potential mechanisms could be excluded. More recently the papers by Chadwick and Rogers (16), Chadwick and Chang (17), Myers et al (18,19), Klubes et al (20), and of Ardølan et al (21) have provided strong evidence that the persistence or peak concentrations of FdUMP in tumors correlated with antitumor effectiveness of 5-FU, and that the antitumor selectivity of the drug could also be explained by the longer retention of this drug metabolite in tumor than in normal tissues such as bone marrow and intestine. Indeed, at these meetings last year (22), we presented evidence of the longer persistence of FdUMP levels in the drug-sensitive mouse solid L1210 tumor than in the relatively insensitive rat solid Walker 256 tumor (fig 2). In adddition, the antitumor selectivity in the mouse was associated with the slower recovery of the rate of incorporation of deoxyuridine into DNA by the sensitive L1210 tumor than by bone marrow and gut; the reverse sequence was observed in the rat, where recovery in the insensitive Walker tumor actually preceded that in the normal host tissues studied. These results are most compatible with the retention of FdUMP as the major target cell determinant of antitumor effectiveness and toxicity (20).

5-FU Incorporated Into RNA

Other information indicates that, at least under certain conditions, the formation of FdUMP and inhibi-

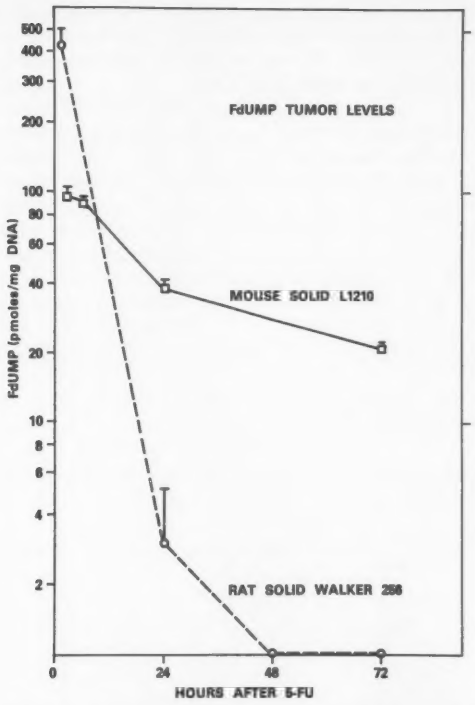

FIGURE 2.—Tumor concentrations of FdUMP in tissue samples from animals after treatment with 5-FU. Mice implanted sc with the 5-FU-sensitive L1210 tumor received 5-FU (100 mg/kg) ip 6 days later. Similarly, rats were injected with Walker 256 cells (relatively insensitive to 5-FU) sc, and after 5 days received 5-FU (120 mg/kg) sc. Acid-soluble FdUMP was extracted from excised tumors at selected times following 5-FU treatment and assayed using *L casei* thymidylate synthetase spectrophotometrically, as described (22).

tion of DNA synthesis do not wholly explain the actions of 5-FU. This compound is not a phase-specific cell cycle-dependent drug, as would be anticipated for an antitumor agent whose sole action is to block DNA synthesis (23). In addition, there are instances where thymidine was ineffective but uridine was effective in abolishing actions of 5-FU in vitro. These observations imply the likelihood of more than one mechanism of pharmacologic activity for 5-FU.

Application of Rescue Agents—Reversal Studies

It would be expected that uridine could block the RNA effects of 5-FU while thymidine, by repleting the diminished pool of thymidylate because of FdUMP-induced inhibition of thymidylate synthetase, would overcome the inhibition of DNA synthesis. However, these studies are complicated by many factors that often make their interpretation ambiguous. These normal pyrimidines may also compete for the anabolism of 5-FU or FUdR, and they may block the degradation of 5-FU and its derivatives. The latter effect is particularly likely in in vivo experiments, much less so in tumor tissue in vitro (24). The various parameters will differ depending on the particular tumor being examined. For example, thymidine reversed FUdR toxicity in HEP-1 cells in culture, suggesting that the dominant cytotoxic pathway for FUdR involved thymidylate synthetase, but it did not prevent the actions of 5-FU, suggesting that in this tumor the formation of FU-RNA was of greater importance (25). On the other hand, the inhibitory effects of 5-FU in vitro on L cells (26), HeLa cells (27), or Ehrlich ascites cells (28) were reversed by thymidine.

The ability of thymidine to reverse the effects of 5-FU may also depend on the concentration of the analog as well as the time of addition of the rescue agent. In the Novikoff tumor, for example, 100 μM thymidine overcame the growth inhibition produced by 1 μM 5-FU, but not that caused by 10 μM 5-FU (29). A delay in the addition of thymidine decreased the effectiveness of this rescue agent (30), which may relate to the gradual loss of thymidine kinase in 5-FU-treated cells (31), thereby preventing thymidine anabolism and bringing about irreversible damage.

Applications of Rescue Agents—Potentiation

In contrast, the investigations of Martin and colleagues (15,31–33), using 5-FU together with thymidine against murine breast tumors in vivo, demonstrated enhanced chemotherapeutic effects. It was postulated at first that the synergism was based on an effect of 5-FU involving mainly RNA. It now appears more probable that thymidine, which is degraded via dihydrothymine, may competitively interfere with the corresponding degradation of 5-FU to dihydrofluorouracil, thereby permitting more of the drug to be anabolized to its cytotoxic derivatives (34). Careful study of model systems where thymidine supplementation apparently enhances the actions of 5-FU indicates such a mechanism, suggesting that the supplementation with thymidine may mimic a higher dose of 5-FU alone (35,36). Thymidine has also been shown to enhance cytotoxicity to AKR leukemia cells in vivo (37), as was first observed for L1210 cells by Burchenal et al. (38).

Applications of Rescue Agents—Tools for Discrimination

In addition, thymidine supplementation of 5-FU-treated bacterial and tumor cells in vitro has permitted the dissociation of the drug's actions on DNA and

RNA synthesis, thereby focusing on drug effects not associated with inhibition of DNA synthesis.

Microorganisms

In our earlier work using exponential cultures of *Bacillus cereus*, inhibition of growth or of cell division produced by 5-FU was not reversed by the supplementation of thymidine (fig 3) (39,40). (Growth was assayed turbidimetrically, which largely measured changes in bacterial cell mass.) However, DNA synthesis for the same increment of growth was comparable to that of normal cells when thymidine was coadministered with 5-FU. In other words, it was possible to demonstrate growth-inhibitory effects of 5-FU even in the absence of an effect on DNA synthesis. Under these conditions, ribosome formation was inhibited (41), and 5-FU was shown to be incorporated into RNA at an increased amount per unit of cell mass[3] (fig 4). Cells harvested from such cultures consequently had an enhanced replacement of uracil by 5-FU, as shown in table 1. The mechanism for this increased incorporation of 5-FU into RNA is unclear, but total RNA synthesis was unchanged under these conditions. Furthermore, in these bacterial cells, prevention of 5-FU degradation by thymidine was not believed to play a significant role. The inhibitory effects on growth by the 5-FU-thymidine combination therefore are probably due to the formation of FU-RNA and/or an effect on ribosome formation or maturation (which cannot be readily dissociated from an RNA effect of 5-FU (13).

It is of interest that in this bacterial system, the failure of cell division in the presence of 5-FU with thymidine led to loss of viability of the cells (fig 5) (40). Growth in the absence of adequate DNA synthesis, which results from FdUMP formation when no thymidine is added, also led to cell death (42).

In this bacterial system, the further addition of uracil to cultures growing in the presence of 5-FU and thymidine permitted a return to normal growth, cell

[3]Doolittle CH, Hahn GA, and Mandel HG. Unpublished observations.

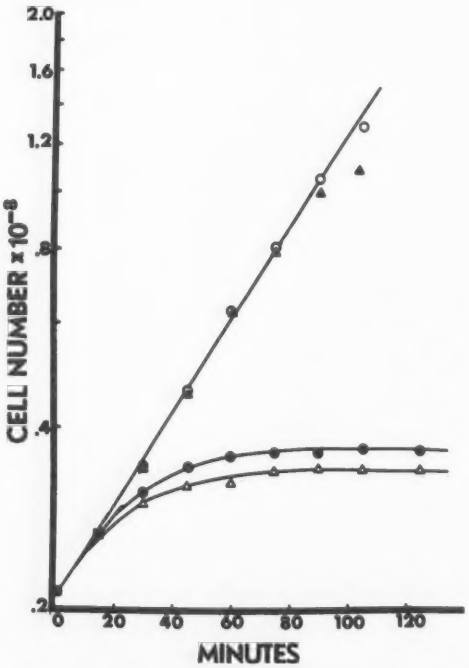

FIGURE 3.—The effect of 5-FU and pyrimidines on cell division in *Bacillus cereus*. Cell counts/ml of culture, recorded with a Coulter cell counter, are plotted against time. Cultures: Control o; 16 μM 5-FU, △; 16 μM 5-FU + 16 μM thymidine (TdR) (repeated at 15-min intervals), •; 16 μM 5-FU + 16 μM TdR + 160 μM uracil, ▲. For details, *see* (39).

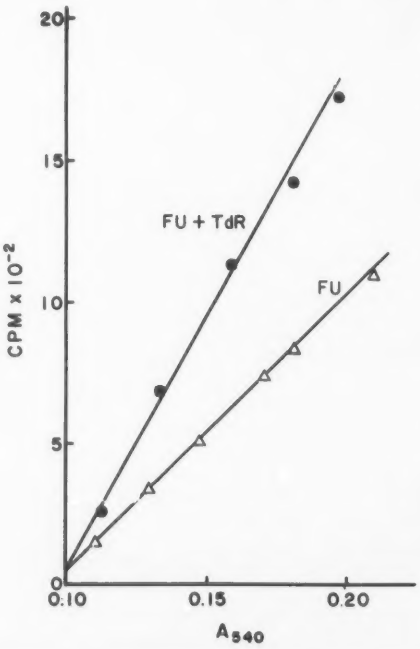

FIGURE 4.—Incorporation of ^{14}C-5-FU into RNA in *B cereus*. Growth was measured turbidimetrically at 540 nm and incorporation monitored by membrane filtration and scintillation counting. Cultures: 16 μM 5-FU, △; 16 μM 5-FU + 16 μM thymidine (TdR) (repeated as in fig 3), •. (Doolittle CA, Hahn GA, and Mandel HG: unpublished observations).

TABLE 1.—Effect of 5-FU in the presence or absence of pyrimidines in cultures of *B cereus**

Treatment	Doubling time (min)	DNA synthesis (% of control)	RNA synthesis (% of control)	U Replacement by 5-FU (%)	Distribution of label in ribosomes (%)
Control	50	100	100	0	75.1±4.0
5-FU	120	26±2	106±15	19.5	32.2±3.0
5-FU + TdR	120	102±3	108±9	46	19.5±3.3
5FU + TdR + U	50	99±3	101±11	5.5	74.1±1.8

*Bacteria were grown, as indicated, in the presence of 5-FU, uracil (U) and thymidine (TdR), and growth was measured turbidimetrically. DNA synthesis was calculated after measuring incorporation of ^{14}C-guanine into DNA and of ^{14}C-guanine into total RNA by membrane filtration, and comparisons were made for equal increases in total cell mass. Base replacement was evaluated from ^{14}C-5FU incorporation data. For the subcellular experiments, cell-free extracts from cultures whose RNA was labeled by growth in the presence of ^{14}C-guanine were prepared and separated on sucrose gradients. Distribution of radioactivity in newly formed RNA between ribosomal and soluble fractions was then calculated ± SEM. Concentrations: 5-FU, 48 μM; TdR, 160 μM; U, 48 μM. [Adapted from (41).]

division (fig 3) and viability (fig 5), indicating that both uracil and thymidine were required to overcome the two very distinct effects of 5-FU (40).

Thus there appear to be two separate determinants of 5-FU action in this bacterial system, both of which can be lethal. The inhibition of DNA synthesis, undoubtedly mediated through the formation of FdUMP, is known to reduce viability by "thymineless death." The presence of exogenous thymidine masks this effect, and the increased formation of FU-RNA emphasizes the second lethal response of 5-FU, which in this model system is correctable with uracil.

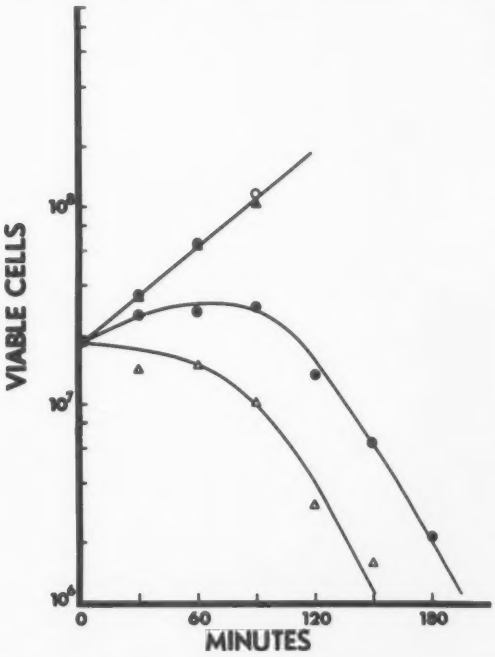

FIGURE 5.—Bactericidal effects of 5-FU in presence or absence of thymidine (TdR) and reversal by uracil (U) plus thymidine in *B cereus*. Viability was determined by pour plate technique, and is plotted against time. Cultures as in fig 3: control, o; FU + TdR, •; FU + TdR + U, ▲. For details *see* (40).

Tumor cells

Several additional developments lend further support to such a dual mechanism of growth inhibition of 5-FU. Wilkinson and Crumley (43), using Novikoff tumor cells in culture, have carried out studies corresponding to the bacterial experiments just described. Fluorouridine (FUR), which was inhibitory to these cells, also produced the expected depression of DNA synthesis and of ribosomal maturation, but not of total RNA synthesis as measured by deoxyguanosine (GdR) degradation and incorporation into the appropriate nucleic acid. Supplementation of the culture medium containing FUR with thymidine prevented the inhibition of DNA synthesis, but growth and ribosomal maturation remained inhibited (table 2). These results are analogous to those observed in the *B cereus* system and have interpreted to involve an important role of FU-RNA in the drug's cytotoxicity to tumor cells. In the absence of exogenous thymidine, the action of 5-FU in inhibiting DNA formation is still recognized as being of grave consequence to the cell. Although the effects of thymidine supplementation on 5-FU incorporation into RNA were not meaured in these experiments, a recent report indicates that FU-RNA remained constant (44).

We have just completed similar measurements on mouse T lymphoma (S-49) cells in culture that are extremely sensitive to 5-FU[4]. The addition of thymidine conferred a 24-hour delay in the onset of growth inhibi-

[4]Maybaum J, Ullman B, Mandel HG, et al. Unpublished observations at the time of the Symposium. Subsequently published (Maybaum J, Ullman B, Mandel HG, et al. Regulation of RNA- and DNA-directed actions of 5-fluoropyrimidines in mouse T-lymphoma (S-49) cells. Cancer Res 40: 4209-4215, 1980.).

TABLE 2.—Effect of FUR in the presence or absence of pyrimidine compounds in Novikoff hepatoma cells in culture*

Additions	Cell division (% of control)	GdR incorporation into DNA (% of control)	GdR incorporation into RNA (% of control)	rRNA maturation
None	100	100	100	Normal
FUR	3	12	104	Inhibited
FUR + TdR	7	104	109	Inhibited
FUR + TdR + U	89	—	—	—

*Suspension cultures of Novikoff hepatoma cells were treated with FUR (10 μM) and pyrimidines for 1 hour, the drug washed out, and cell concentration, isotope incorporation, and rRNA maturation measured as described (43). Concentrations of thymidine (TdR) and uridine (U) were 100 μM.

tion by 5-FU but thereafter it had little influence on the rate of cell proliferation. The incorporation of 5-FU into RNA was enhanced by the presence of thymidine, while DNA synthesis returned toward that of control cells (table 3).

The enhancement by thymidine of the incorporation of 5-FU into RNA of tumor cells has already been observed by Nayak et al (33), who reported up to 40-fold increases in incorporation of 5-FU into nuclear RNA of tumor-bearing mice. Carrico and Glazer (45) have observed enhanced incorporation of 5-FU into RNA fractions by thymidine coadministration in regenerating rat liver following hepatectomy. Since very little DNA synthesis takes place under the time conditions chosen for these experiments, this system is useful in discriminating between RNA and DNA effects. As shown in table 4, the addition of thymidine increased the amount of 5-FU recovered in ribosomal RNA and poly(A)RNA whereas the utilization of orotate, representing a measure of total RNA synthesis in these fractions, was actually somewhat diminished under such conditions. It was concluded that thymidine pretreatment intensified the effect of 5-FU on interference with the processing of ribosomal RNA. It is not known from these in vivo studies whether the mechanism of enhanced incorporation of 5-FU was due to decreased degradation of 5-FU in the presence of thymidine.

Pharmacologic Correlations With FU-RNA Formation

Additional evidence of the importance of a 5-FU-induced RNA effect may come from the correlations of increased FU-RNA formation and enhanced tumor sensitivity to 5-FU. A tendency for such a relationship was established for a small number of tumors, but other biochemical effects, such as FdUMP formation, correlated equally well (9). For a series of transplanted mouse tumors, Kessel et al (46) found a remarkable relation between the ability to convert 5-FU to phosphorylated derivatives and the sensitivity of such tumors to 5-FU. The phosphorylated fraction would be expected to contain acid-soluble products such as 5-fluorouridine triphosphate (FUTP) and, presumably free FdUMP. About 40% of the total was said to be in the form of insoluble derivatives, representing mainly FU-RNA and probably a trace of bound FdUMP. The survival times of tumor-bearing animals treated with 5-FU appear as a direct function of drug anabolism, but

TABLE 3. Effects of 5-FU and thymidine in S-49 cells in culture*

Hours of incubation	[FU]	[TdR]	% growth inhibition	DNA / RNA	5-FU incorporation (μg 5-FU/mg RNA)
72	—	—	—	0.77	—
	0.7 μM	—	55	0.70	0.082
	—	10 μM	17	0.82	—
	0.7 μM	10 μM	39	0.81	0.135
16	—	—	—	—	—
	2.0 μM	—	55	0.66	0.120
	2.0 μM	10 μM	0	0.82	0.148

*Samples were removed from cultures grown with ^{14}C-5-FU and thymidine (TdR) as indicated, and extracted with perchloric acid. The insoluble residue was incubated with 0.3N KOH overnight, neutralized, and centrifuged. The liberated ribomononucleotides in the supernatant were quantitated spectrophotometrically, and 5-FU incorporation was assayed by scintillation counting. DNA was measured in the residue by colorimetric assay. (Maybaum J, Ullman B, Mandel HG, et al. Unpublished observations).

TABLE 4.—Effect of thymidine on 5-FU incorporation into RNA fractions of regenerating rat liver*

RNA species	5-FU in RNA (pmoles/μg RNA)		Orotate incorporation into RNA (% of control)	
	5-FU	5-FU + TdR	5-FU	5-FU + TdR
rRNA	0.86	2.36	36	21
poly(A)RNA	2.94	10.34	110	73

*Partially hepatectomized rats received labeled 5-FU following thymidine (TdR) or saline pretreatment. Polysomes were isolated from liver and RNA extracted and separate into rRNA and poly(A)RNA by affinity chromatography. Orotate experiments were carried out similarly. From (45).

again unfortunately, the precise product responsible for the effect is not known (fig 6). Laskin et al (47) reported a specific correlation between increased incorporation of 5-FU into RNA of seven mouse or human tumors and response to 5-FU therapy. In other investigations, however, Kessel et al (48) found widely varying concentrations of FU-RNA in spite of similar antitumor responses. In human xenografts, the degree of inhibition of growth by 5-FU and formation of FU-RNA also did not correlate (49).

By means of coadministration of other compounds, perturbations in FU-RNA formation have been produced reflecting changes in tumor sensitivity to 5-FU. For example, inosine by acting as a donor of ribose-1-phosphate, increased the growth inhibition by 5-FU of various tumors while also increasing the incorporation of 5-FU into RNA (44,47). Although there was no evidence of a corresponding enhancement of FdUMP formation, the inability to detect this metabolite even in the absence of inosine does not rule out such a possibility. Similarly, glucose supplementation of 5-FU increased FU-RNA formation in vivo and in vitro in various tumors while producing an enhanced antitumor response (50), but this effect was not consistently observed (51). These studies can be summarized as indicating a correlation between FU-RNA formation and antitumor sensitivity to 5-FU in certain systems but clearly not in others. Thus, FU-RNA probably contributes to, but is infrequently the sole determinant of susceptibility to 5-FU effectiveness.

The recent report of Houghton et al (52) represents another approach that emphasizes the potential importance of FU-RNA formation as a determinant of drug action. Using labeled 5-FU, FUR, or FUdR, which were given at equitoxic doses to mice, these authors found a close quantitative relationship between the specific activities of FU-RNA in gastrointestinal mucosa tissues, regardless of which labeled 5-FU compound was administered. On the other hand, the corresponding measurement of FdUMP levels provided much greater discrepancies (table 5). The investigators concluded that drug lethality was due to gastric toxicity.

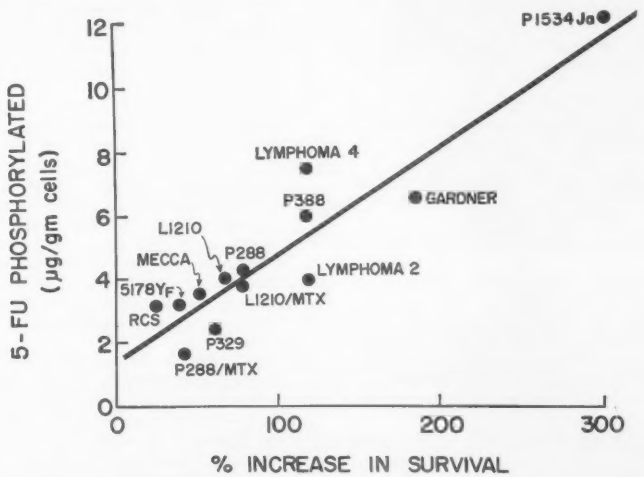

FIGURE 6.—Correlation between extent of anabolism of 5-FU to inhibitory compounds by phosphorylation in a series of animal tumors, and increase in survival time of tumor-bearing animals following treatment with 5-FU. From (46).

TABLE 5.—Ratios of [FdUMP] and FU-RNA Specific Activities in Mouse Tissues After Equitoxic Doses of ^{3}H-FU Compounds*

Tissue	Ratio, [FdUMP] in tissues (moles/g tissue)			Ratio, FU-RNA (moles/mg RNA)		
	^{3}H FUR	^{3}H FU	^{3}H FUdR	^{3}H FUR	^{3}H FU	^{3}H FUDR
Stomach	1	3	3.9	1	0.9	1.3
Duodenum	1	5.7	6	1	1.3	1.2
Ileum	1	9.0	17.3	1	1.3	0.8
Colon-rectum	1	13	18	1	2.2	0.95
Mean	1	7.7	11.3	1	1.4	1.06

*Mice received doses of labeled 5-FU compounds which were shown to produce a similar degree of toxicity. GI tissue was then examined for FU-RNA and also for FdUMP isolated chromatographically. From (52).

CONCLUSION

There is increasing evidence that 5-FU produces its antitumor effects by more than one mechanism. By using means to dissociate these drug effects it is now possible to attach importance to the effect of FdUMP on DNA synthesis and to the incorporation of 5-FU into RNA. The relative roles of these two drug effects have been shown to vary, depending on the biological system being studied and the experimental conditions being used, but either or both can be considered capable of acting as the critical determinant(s) of action of 5-FU.

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Determinants of Response to Antimetabolites and Their Modulation by Normal Purine and Pyrimidine Metabolites[1]

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SUMMARY

Two areas of investigation are discussed: (*a*) the identification of critical biochemical parameters that may lead to prediction of response of tumor cells to antimetabolites, such as 1-β-D-arabinofuranosyl-cytosine (ara-C) and 5-FU, and (*b*) the potential use of normal purine and pyrimidine metabolites in the selective and specific modulation of critical parameters related to ara-C and 5-FU activity. The results presented indicate a strong correlation between ara-CTP formation and retention in leukemic cells and response of animals with leukemias or patients with acute myelocytic leukemia (AML) treated with ara-C or a protocol containing ara-C. Furthermore, thymidine was found to be an effective modulator of the intracellular pools of dCTP and ara-CTP in rats, bone marrow, small intestine, and colon tumor cells. The mechanism of this effect is unclear, but initial evidence indicates that specific scheduling of the sequential administration of the metabolite and the antimetabolites may be important in determining increased selectivity of antitumor effects. In vivo studies with fluorinated pyrimidines have focused on quantitating 5-fluorodeoxyuridine monophosphate (FdUMP) pools and their retention, and on the incorporation of fluorouridine (FUR) into RNA of sensitive and resistant L1210 cells. The results suggest that the retention of intracellular FdUMP pools above a threshold may be a more critical determinant in selectivity than the initial FdUMP levels achieved in target cells. Thymidine has been demonstrated to alter the pharmacokinetic parameters of 5-FU in man and in mice, but a selective modification of the antitumor activity of 5-FU has not yet been unequivocally demonstrated.

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Despite nearly three decades of widespread and highly sophisticated biochemical and pharmacological investigations of 5-FU and ara-C, some aspects of the mechanisms of action of these agents are still unclear, especially as they may be related to predicting clinical efficacy. In general, the lethal action of these drugs in target cells depends on numerous interactions among critical factors operating in the whole organism as well as in the target cells themselves. Although it is possible that lack of uptake of these drugs (including transport and activation) may be sufficient to predict resistance, uptake per se is unlikely to be adequate to predict response. Factors such as the intracellular concentrations and retention of the active form of these drugs, the intracellular concentrations of competing metabolites, the presence of metabolically susceptible sites, and the levels of activating and deactivating enzymes may all play a role in determining the degree of selectivity of these agents. In this presentation, the identification of probable determinants of the action of ara-C and 5-FU and the role of normal metabolites in modulating the activity of these two drugs are discussed.

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DETERMINANTS OF RESPONSE TO 5-FU AND THEIR MODULATION BY NORMAL PURINE AND PYRIMIDINE METABOLITES

Although treatments with 5-FU have been mostly palliative, producing some regressions of tumor with extensions in survival, this drug seems to be rarely, if ever, curative. Irrespective of the schedule and route of administration, the incidence of clinical response to 5-FU in colonic carcinoma rarely exceeds 20%. Therefore, it would be important to be able to identify the factors that determine response to this drug and thus possibly improve its utilization in the treatment of human cancer.

5-FU was synthesized in 1957 (1) and shortly thereafter was shown to have tumor inhibitory activity (2,3). As demonstrated initially by Heidelberger (2–4), 5-FU, 5-fluorouridine (FUR), and 5-fluorodeoxyuridine (FUdR) are metabolized in mammalian cells via the so-called salvage pathways normally available to exogenous or metabolically produced pyrimidines. Figure 1 shows the possible points of attack of the fluorinated pyrimidines and also indicates how thymidine (TdR) may interfere with the pharmacokinetics and intracellular metabolism of 5-FU and its derivatives. It can be seen that 5-FU may be channeled either in the direction of DNA or RNA synthesis depending on the relative significance of thymidine or uridine phosphorylase, respectively. The first pathway leads to the formation of FdUMP which is a potent inhibitor of thymidylate synthetase (TMPS) (4), the enzyme responsible for the methylation of deoxyuridine monophosphate (dUMP) to thymidylate (TMP), which is essential in the de novo synthesis of DNA. It is generally accepted that the inhibition of TMPS is responsible for the carcinostatic action of 5-FU (4). If 5-FU is ribosylated by uridine phosphorylase, it may ultimately be incorporated into RNA or be reduced at the diphosphate level to the corresponding deoxyribose analog and subsequently dephosphorylated to yield FdUMP. It should be added that the initial steps in the above pathway may be bypassed through the action of phosphoribosyl transferase, which directly converts 5-FU to FUMP. The incorporation of fluorinated uridine into RNA has been demonstrated and has been considered by some to have at least a partial role in the cytotoxic effects of these drugs (5,6). Until recently, however, the possible consequences of the latter effect have received far less attention than the effect on DNA synthesis via inhibition of thymidylate synthetase. The catabolic conversion of 5-FU into the inactive metabolite, dihydrofluorouracil (FUH_2), is rapid and saturable. This inactivation, which accounts for the elimination of over 80% of the 5-FU dose, may be significantly altered by the coadministration of inhibitors of dihydrouracil dehydrogenase.

In humans, TdR is rapidly converted to thymine (T) and deoxyribose 1-phosphate (dR-1-P) (7); T may then

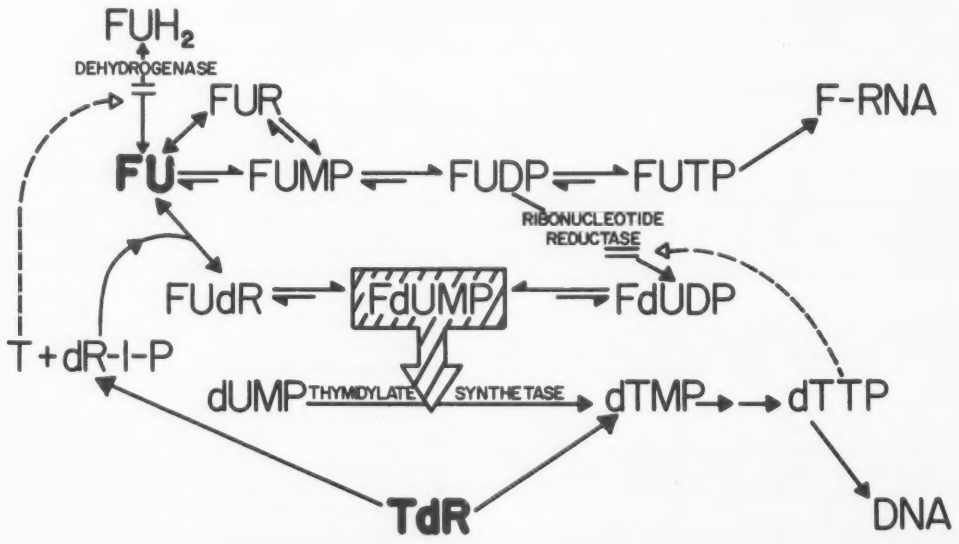

FIGURE 1.—Metabolism of F-pyrimidines and TdR. FUMP, FUDP, and FUTP are the 5'- mono, di-, and triphosphate of 5-FU, respectively; dUMP, deoxyuridine 5'-monophosphate; TMP and dTTP, 5'-mono-, and triphosphate of TdR, respectively.

competitively inhibit the conversion of 5-FU to FUH_2, resulting in higher plasma concentrations of the drug with subsequent conversion of 5-FU to FUdR due to the availability of dR-1-P. The cellular selectivity of 5-FU under the condition of TdR coadministration may depend on the degree of modulation of the catabolic and anabolic pathways of 5-FU by TdR in the target cells.

Determinants of Response to 5-FU

Studies have been carried out in this laboratory to quantitate the differences that may exist between L1210 sensitive (L1210/0) and resistant (L1210/FU) to 5-FU in the formation and retention of FdUMP and in the incorporation of 5-FU into RNA. Results obtained in preliminary experiments indicated that while 5-FU and FUdR produced a 20%–60% increase in life span (ILS) of DBA/2J mice bearing L1210/0, FUR caused a 200% ILS. L1210/FU cells were resistant to all three F-pyrimidines.

Due to the large difference in the sensitivity of the two leukemia lines to FUR, studies were carried out in vivo using ^{3}H-FUR. The formation of FdUMP and the incorporation of radioactivity into RNA were determined at various times following ip administration of a single dose of FUR containing ^{3}H-FUR. The results summarized in table 1 indicate that the FdUMP levels and the amount of drug incorporated into RNA were both several fold higher in sensitive than in resistant cells and that the intracellular retention of FdUMP was greater in L1210/0 than in L1210/FU cells. For example, 24 hr after the administration of the drug, 12 pmol/10^7 cells of FdUMP was detected in sensitive cells while none was detected in resistant cells. Using the same approach but comparing L1210/0 with P-288 cells, which are naturally resistant to 5-FU (data not shown), the incorporation into RNA was not significantly different between the two cell lines while the FdUMP pools and their retention were significantly higher in L1210/0 than in P-288 cells. These data suggest that, at least in some cell lines, the FdUMP pools and their retention may be more critical to therapeutic response than the incorporation into RNA. This conclusion may vary depending on the cell type examined. Studies are underway using human and rodent tumors to verify this possibility.

In order to further understand the biochemical basis of tumor cell sensitivity to F-pyrimidines, attempts have been made to correlate the initial pools of FdUMP with the degree of inhibition of TMPS. The data in table 2 show the in vitro effects of 5-FU, FUR, and FUdR on the activity of TMPS in sensitive and resistant L1210 cells. After a 2-hr exposure of L1210/0 to FUR and FUdR, TMPS was inhibited about equally at all concentrations, while comparable inhibition of TMPS by 5-FU was only observed at 10^{-5} M. Little inhibition of TMPS by 5-FU or FUR was observed in L1210/FU at drug concentrations of 10^{-6} M. In contrast, FUdR was equally effective in inhibiting TMPS in both sensitive and resistant cells. It should be stressed that although in vitro the TMPS activity of L1210/0 was inhibited to <10% of control by FUdR, this agent (200 mg/kg x 1 day or 50 mg/kg x 5 days) produced no more than a 20%–60% ILS of DBA/2J mice inoculated with 10^6 tumor cells ip. This observation, together with the lack of response of L1210/FU to FUdR in spite of the dramatic reduction of TMPS activity at 10^{-7} M FUdR, suggests that other factors in addition to the inhibition of TMPS may also be critical to the sensitivity of certain tumor cell lines. One such factor may be the retention of FdUMP above a critical level to effectively inhibit the TMPS. In addition, the time course of such inhibition may also contribute to the antitumor activity.

In conclusion, the data presented in table 1 and those reported previously (9) indicate that high initial pools of FdUMP derived from FUR and the retention of the FdUMP pool may be critical determinants of reponse of certain tumor cells. Similar conclusions have been reached by Klubes et al (10). Therefore, when the initial pools of FdUMP fall below a critical threshold, as is the case in L1210/FU, it should be possible to predict resistance. Further studies using tumor cells sensitive and resistant to 5-FU will be carried out to quantitate the pools of FdUMP and the incorporation of

TABLE 1.—Intracellular FdUMP and F-RNA levels after FUR administration*

Tumors	FdUMP				F-RNA		
	1 hr	4 hr	24 hr	% Retention	1 hr	4 hr	24 hr
	(pmol/10^7 cells)			at 4 hr	(pmol/10^7 cells)		
L1210/0	230	95	12	41	2019	1880	102
L1210/FU	30	6	< 1	20	310	260	12
S/R†	7.7	10.5	>12	2	6.5	7.2	8.5

*Mice bearing tumors sensitive (L1210/0) or resistant (L1210/FU) to 5-FU were injected ip with FUR (10 mg/kg containing 100 μCi ^{3}H-FUR). At 1, 4, and 24 hr after administration of the drug, cells were removed, washed, centrifuged, and acid-soluble and RNA fractions were prepared.
†Ratio of sensitive to resistant.

TABLE 2.—Effects of F-pyrimidines on thymidylate synthetase activity*

Drugs	Tumors	Drug concentration (M) 10^{-5}	10^{-6}	10^{-7}
		(% of controls)		
5-FU	L1210/0	28	62	79
FUR	L1210/0	14	17	7
FUdR	L1210/0	8	10	10
5-FU	L1210/FU	69	96	81
FUR	L1210/FU	67	99	115
FUdR	L1210/FU	4	10	12

*L1210/0 and L1210/FU cells were incubated with F-pyrimidine for 2 hr, then washed, extracted, and assayed for thymidylate synthetase activity (8).

F-pyrimidines into RNA at various times after the administration of a therapeutic dose of the drug and to correlate the pools of FdUMP and their retention with the extent of TMPS blockade and recovery on one hand, and with therapeutic response on the other hand. Only after these types of investigations are carried out in animals and in humans, may it become possible to identify the sets of determinants, that are critical for drug action in different tumors.

Modulation of 5-FU Response by Normal Metabolites

In a previous study (11), the role of uridine (UR), TdR, T, uracil (U), inosine (Ino), deoxyinosine (dIno), and deoxyuridine (UdR) in the in vivo modulation of F-pyrimidine metabolism was investigated. The results can be summarized as follows. Pyrimidine bases supplied exogenously or derived from deoxy- or ribonucleosides can reduce plasma levels of FUH_2 derived from 5-FU, FUR, or FUdR. In addition to this sparing of 5-FU, the drug may be channeled via the ribo- or deoxyribonucleoside pathway depending on the nucleoside employed (fig 1). For example, when UR was used in combination with 5-FU, the major compounds found in plasma were FUR and 5-FU. When UdR or TdR was used, FUdR and 5-FU were the predominant components. Under the conditions employed (metabolites-antimetabolites combination), metabolites may potentiate the toxicity of 5-FU depending on the concentration of the metabolite used and its route of administration. Following the administration of nucleoside metabolites, the nucleoside/base ratio derived in vivo is also an important factor. It was found that potentiation of the in vivo toxicity of 5-FU by the metabolites studied was in the order of UR > Ino > TdR > UdR > dIno.

The antitumor activity of 5-FU in combination with TdR was evaluated against mouse colon tumors 26 and 38 of Corbett et al (12) and against rats bearing colon carcinoma (13). The concurrent combination of TdR (5 mg/kg/72 hr) with the maximally tolerated dose (MTD) of 5-FU (100 mg/kg/72 hr) did not significantly increase the inhibition of tumor growth nor the survival time of mice or rats bearing these tumors over that observed with 5-FU alone at the MTD [150] mg/kg/72 hr). At equimolar doses below the MTD, however, TdR in combination with 5-FU produced greater inhibition of tumor growth in both tumor cell lines in mice as well as in rats. Similarly, results of a phase I-II trial with continuous infusion of 5-FU and TdR in the treatment of colon carcinomas indicate no significant improvement in the response rate over what would normally be expected from 5-FU alone (14).

The plasma levels of TdR and T achieved during continuous infusion of TdR in mice, rats, and humans were measured and are compared in table 3. These results show significant interspecies differences in the extent of TdR conversion to T, with the most extensive conversion being observed in humans. Furthermore, the TdR/T plasma level ratio in rats decreased seven-fold when the dose of TdR was increased from 1.7 to 10 g/kg/day, indicating nonlinear kinetics in the TdR conversion to T. The pharmacokinetic interactions between TdR and 5-FU were investigated in cancer patients. There was a linear correlation between the plasma levels of T and of 5-FU following continuous infusion of 5-FU (7.5 mg/kg/day x 5) and TdR (8 g/m²/day x 6), preceded by iv bolus of TdR (15 g/m²) (7). Pharmacokinetic analysis of the data indicates that the clearance of 5-FU in the presence of 20 μM and 300 μM T plasma concentration was reduced to 25% and 5%, respectively, of the literature value when 5-FU was infused alone (15). Thus, the extent of the modification of the pharmacokinetic parameters of 5-FU by TdR would be dependent on the TdR conversion to T. The large interspecies difference in the ratios of plasma concentrations of TdR/T may partly account for the observed differences in antitumor activity of 5-FU/TdR combination in mice, rats, and humans.

In addition to characterizing the modulation of 5-FU pharmacokinetics by TdR, the effects of TdR (at

TABLE 3.—Plasma levels of TdR and T during TdR infusion*

	Mouse†	Rat‡		Human
	Dose§ 1.7	1.7	10	0.22
Compound		μM		
TdR	66.1	100	490	32.10
T	10.3	5	180	408.70
TdR/T	6.4	20	2.7	0.08

*The values for plasma levels represent the average of 3, 10, and 33 individual determinations for mouse, rat and human, respectively.
†Balb/c mice
‡Fischer rats
§In gm/kg/day

various concentrations and times) on the metabolism of 5-FU and FUdR in tumor cells have also been examined. The data in table 4 outline the results obtained using cells from the peripheral blood of leukemic patients. The results are consistent with those previously reported (16) in that: (*a*) over 80% of the acid-soluble pools derived from ^{3}H-FUdR were in the form of FdUMP while $< 10\%$ of the pools derived from ^{3}H-5-FU were in the form of FdUMP; (*b*) in every case, TdR reduced the intracellular pools of FdUMP and the total radioactivity in acid-soluble fractions, consistent with the findings of Bowen et al (17) indicating that the metabolism to FdUMP, but not the transport of FUdR, may be inhibited by TdR; (*c*) there were no consistent patterns as to the effect of TdR on the incorporation of F-pyrimidines into RNA. In some cases, there was an increase while in others there was no effect (data not shown).

Thus, on the whole, TdR did not appear to modify significantly the antitumor activity of 5-FU against human and rodent colon tumors when both agents were administered by continuous infusion (13,14). The toxicity of 5-FU, however, could be dramatically altered in vivo by the coadministration of TdR or other normal metabolites. This is probably, due in part, to decreased catabolism of 5-FU. Preliminary results of biochemical studies using leukemic cells indicate that the effect of TdR on F-pyrimidines is the reduction in the intracellular FdUMP pool with potentiation of F-pyrimidine incorporation into RNA in some cases. The consequences of these effects will be evaluated further in human malignant cells.

DETERMINANTS OF RESPONSE TO ARA-C AND THEIR MODULATION BY TdR

Determinants of Response

The activity of this antimetabolite is apparently specific for the S-phase of the cell cycle (18). The effectiveness of ara-C depends on its phosphorylation by deoxycytidine kinase to the ara-C triphosphate (ara-CTP), which is an inhibitor of DNA polymerase (19,20), and becomes incorporated into nucleic acids (21,22). Pathways for the metabolism of deoxycytidine (CdR) and its analog, ara-C, and of TdR are shown in figure 2.

Initial studies directed towards identifying critical biochemical determinants of ara-C action were carried out in mice using sensitive (leukemia L1210) and relatively resistant (leukemia P288 and Taper

TABLE 4.—Effects of TdR on intracellular FdUMP pools in AML patients*

	pmol/10^7 Cells			
	5-FU		FUdR	
Patients	−TdR	+TdR	−TdR	+TdR
1	11.6	8.3	569.2	112.8
2	2.2	1.3	231.1	61.5
3	1.8	< .3	240.2	18.4
4	6.6	2.1	120.8	40.2

*Cells obtained from the peripheral blood of patients with acute myelogenous leukemia were incubated at 37 °C with and without TdR (10^{-4} M) for 2 hr, washed, and reincubated for 30 min in fresh media (−TdR) in the presence of 4 M ^{3}H-5-FU or ^{3}H-FUdR.

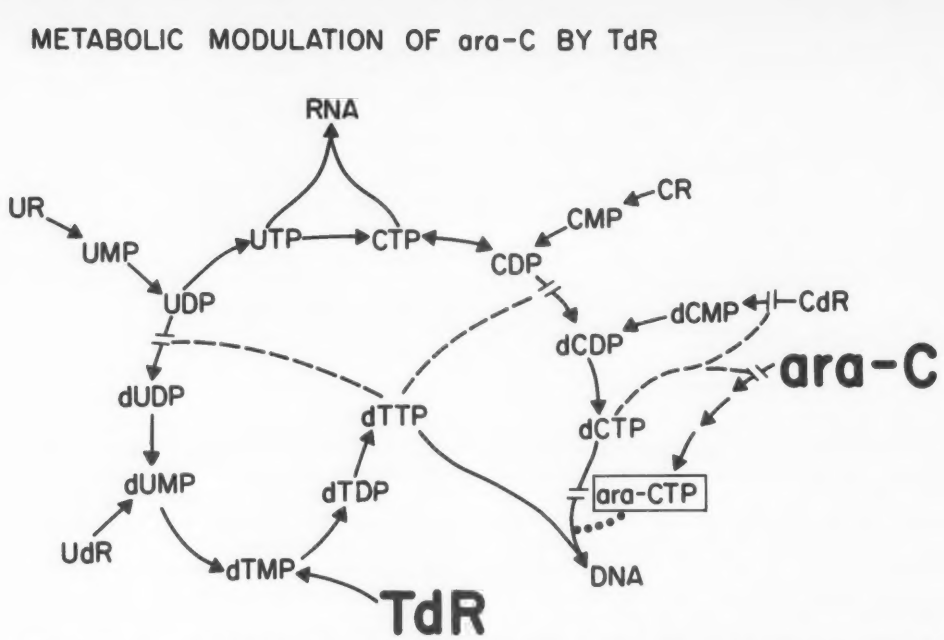

FIGURE 2.—Metabolism of ara-C and TdR. UMP, UDP and UTP are the 5'-mono-, di-, and triphosphate of uridine, respectively; CMP, CDP, and CTP are the 5'-mono-, di-, and triphosphate of cytidine, respectively; dCMP dCDP, and dCTP are the 5'-mono-, di-, and triphosphates of deoxycytidine.

hepatoma) tumors (23). The results of these studies indicated that the formation and retention of ara-CTP intracellular pools are critical determinants of response to ara-C of animals bearing tumors insofar as these parameters correlated directly with the therapeutic effectiveness of the drug. Moreover, it was found that ara-CTP levels were retained longer in the responsive tumors than in host bone marrow and small intestine and this difference appeared to play a critical role in the selectivity of antitumor action of the drug. Quantitation of intracellular metabolites of ara-C and their ratios at various times following an in vivo or in vitro exposure to the drug (23) showed that intracellular deamination of ara-C or ara-UMP was not a critical determinant.

ara-C metabolism was investigated in AML cells obtained from the bone marrow of patients prior to treatment to determine whether or not the parameters identified as predictors of response in the three mouse tumors could be equally predictive in humans (24). In initial studies, the cells were incubated in vitro for 30 or 60 min with different conentrations of ara-C, then were removed, washed, and resuspended in drug-free medium. The intracellular pools of ara-CTP were quantitated at various times. The ability of AML cells to transport and activate ara-C to ara-CTP was directly related to the concentration of ara-C in the incubation medium and to the length of incubation at 37 °C. The data indicated that at all concentrations of ara-C, the formation of ara-CTP was linear for up to 3 hr of incubation. The concentration dependency of the intracellular retention of ara-CTP was also investigated. At 4 hr, the intracellular pools of ara-CTP of cells incubated with 0.125, 0.5, and 1.0 μg/ml ara-C were 38%, 37%, and 41% of the initial values, respectively. These results indicated that the formation of ara-CTP from ara-C, but not the retention of ara-CTP, is dependent on the extracellular concentration of ara-C.

The study of the possible correlation of the intracellular metabolism of ara-C and/or ara-CTP retention to response was carried out using bone marrow blast cells obtained from 28 untreated patients (24). As shown in table 5, in terms of in vitro ara-CTP retention at 4 hr, these patients fell into three groups: group 1, 13 cases with an average retention of 57.5% (with a range of 36% to 73%); group 2, 8 cases where an average retention was 11.6% (with a range of 2% to 19%); and group 3, 7 patients with an ara-CTP level too low to measure at 4 hr (<0.1 pmol/10^7 cells). In one patient in group 3, the initial value of ara-CTP was 1.7 pmol/10^7 cells with no detectable ara-CTP pools at 4 hr. For the purpose of correlating these values with clinical

TABLE 5.—Subclassification of AML patients with no prior chemotherapy on the basis of intracellular ara-CTP formation and retention*

Group	No. of patients	ara-CTP pools (pmol/10^7 cells) 0 hr	4 hr	% retention at 4 hr
1	13	13. ± 9.0	7.4 ± 4.3	57.5 ± 10.5
2	8	2.9 ± 2.1	0.3 ± 0.3	11.6 ± 6.7
3	7	0.1	0.1	TLM†

*ara-CTP formation and retention studies were carried out in the presence of 7.0×10^{-7} M 5-^{3}H-ara-C for 30 min (zero time), after which cells were washed free of extracellular drug, resuspended in fresh medium, and reincubated for 4 hr at 37 °C. Retention was expressed as % of ara-CTP remaining intracellularly at 4 hr following the initial 0 hr incubation.

†Too low to measure.

response to treatment with ara-C and anthracycline, the cases in groups 2 and 3 were considered together (24). Of the 13 patients in group 1, 11 attained complete remission. Seven of these 11 patients remained in complete remission for periods ranging from 132+ to 214+ weeks. Nine out of the 15 patients in groups 2 and 3 attained complete remission. Eight of these patients relapsed between 11 and 92 weeks and one relapsed at about 160 weeks.

It should be noted that in the blast cells from these 28 AML patients, whether from bone marrow or peripheral blood, the intracellular pools of ara-UMP remained relatively constant during the 4-hr incubation in ara-C-free medium during which time the pools of ara-CTP were decreasing. Moreover, over 90% of the label found in the incubation medium at 4 hr was in the form of ara-C. Thus it would appear that the differences in intracellular retention of ara-CTP in vitro could not be accounted for by differences in intracellular deamination. The biochemical mechanisms responsible for the differences in ara-CTP retention among patients require further investigation.

Inhibition of TdR Incorporation into DNA

Initial studies were aimed at developing a functional assay that would aid in evaluating the consequences of adequate ara-CTP pool formation and retention in target cells. The in vitro effects of ara-C at various concentrations (0.0625 to 1.0 μ g/ml) on the incorporation of TdR into DNA were evaluated. At each concentration, ara-CTP pools were quantitated so they could be correlated with the extent of inhibition of TdR incorporation into DNA.

As shown in figure 3, the extent of TdR inhibition seemed to be only moderately dose dependent; for example, at 0.0625 μg/ml of ara-C, this inhibition varied from 5% to 98% in cells from 16 different individuals, while at 1 μg/ml it varied from 22% to 98% in cells from 38 patients. The data also indicated that the greatest spread in the extent of inhibition was observed with ara-C concentrations lower than 0.125 μg/ml, which are close to the plasma concentrations of ara-C achieved in patients during treatment with a standard regimen of ara-C and anthracycline (25). Figure 4 shows the correlations observed between ara-CTP pools and inhibition of TdR incorporation into DNA by ara-C in AML

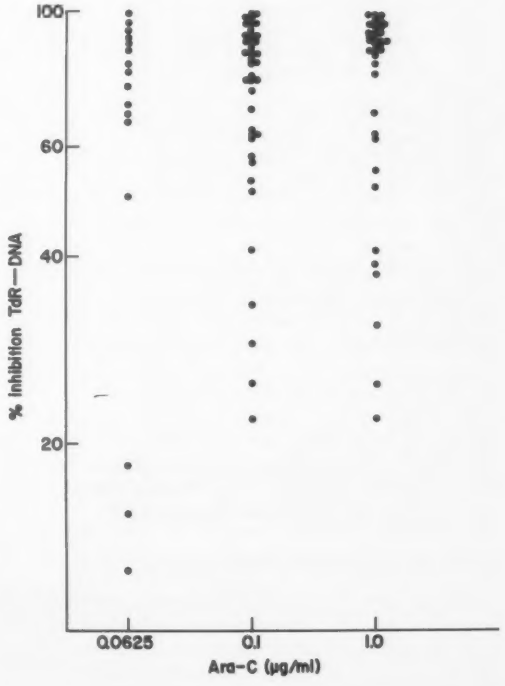

FIGURE 3.—In vitro effects of ara-C on the incorporation of TdR into DNA of AML cells. Cells were exposed to ara-C for 30 min with 15 min exposure to ^{3}H-TdR.

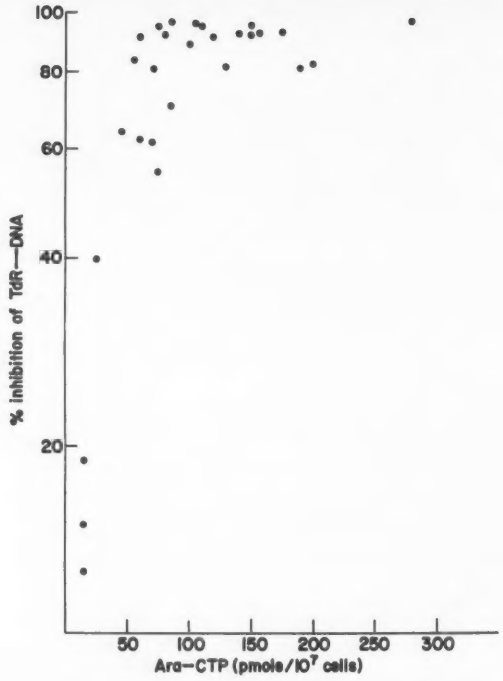

FIGURE 4.—Correlation between ara-CTP pools and inhibition of TdR incorporation into DNA. ara-C inhibition of TdR incorporation into DNA was carried out as outlined in fig 3. ara-CTP pools were quantitated by HPLC on a separate aliquot of cells obtained from the same specimen following a 30-min incubation at 37 °C with 5-^{3}H-ara-C.

cells. There seemed to be a possible linear relationship between percent inhibition of incorporation of TdR into DNA and ara-CTP pools up to about 50 pmol ara-CTP/10^7 cells. However, the percent inhibition of TdR incorporation into DNA reached an asymptote when ara-CTP levels were above 50 pmol/10^7 cells.

The value of this parameter remains to be ascertained. It is particularly important to determine whether or not these types of analyses are affected by the deoxyribonucleotide pool fiuctuations related to experimental conditions and/or chemotherapy.

Modulation of ara-C Response by TdR

Studies have been initiated in rats using transplanted tumor lines derived from a chemically-induced colon tumor to assess the potential value of TdR in determining the selective modulation of the tissue distribution, activation, and cellular retention of ara-CTP. As outlined in figure 2, dTTP may interfere with the formation of intracellular pools of dCTP, the normal metabolite competing with ara-CTP for DNA polymerase inhibition. This interference will undoubtedly depend on the intracellular steady-state level of dTTP. A continuous intravenous infusion system for rats (26) permits infusion of unrestrained animals for up to 1 week.

Several effects were observed when TdR was administered by continuous infusion that could not be observed when the compound was given by a single iv bolus injection (27). Within 3 hr after the initiation of the infusion, a steady-state plasma level of 100 μM TdR was achieved and was sufficient to produce maximum reduction of CdR from 21.8 $\pm$ 4.6 to 4.4 $\pm$ 0.6 μM. CdR reduction was dependent on the TdR dose but recovery was observed within 6 hr after the end of TdR infusion. Pretreatment with a 24-hr infusion of TdR (7 g/kg/day) followed by concomitant infusion of TdR and ara-C for an additional 48 hr reduced the maximally tolerated dose of ara-C from 225 to 7.5 mg/kg/day, a 35-fold increase in ara-C toxicity. In contrast, a 24-hr continuous infusion of TdR followed by a 48-hr continuous infusion of ara-C alone reduced the maximally tolerated dose of ara-C from 225 to 175 mg/kg/day. Under the condition of CdR reduction consequent to TdR infusion, a concomitant increase in the uptake, activation, and incorporation of ara-C into nucleic acids was observed. The tissue increase in ara-CTP pools was in the order of bone marrow > small intestines > tumor. Preliminary results indicate that the pools of ara-CTP remained relatively unchanged in tumor tissue as a function of time; if ara-C administration after the termination of TdR infusion was delayed for 3 hr, ara-CTP pools in the bone marrow and small intestines were reduced dramatically. The best tumor growth inhibition observed thus far was obtained by the sequential administration of TdR prior to ara-C alone given at the maximally tolerated dose. Under these conditions, the antitumor activity of ara-C against the chemically-induced rat colon tumor appeared to be directly related to the dose of TdR infused prior to ara-C infusion.

The data summarized in figure 5 show the effects of different doses of TdR infused over 24 hr followed by a 48-hr infusion of ara-C on tumor growth inhibition and circulating CdR levels in the rats. These data indicated that maximum reduction of CdR in plasma was obtained at TdR doses > 0.5 g/kg/day. The results also indicated that although the circulating CdR level was not modified drastically by increasing the dose of TdR beyond 0.5 g/kg/day, greater tumor growth inhibition was observed with increases in the amount of TdR infused. Although TdR alone reduces CdR levels in rat plasma, infusion of TdR up to 7 g/kg/day for 72 hr had an insignificant effect upon tumor growth.

These results provide evidence for an in vivo modulation of a metabolite, CdR, by another metabolite, TdR,

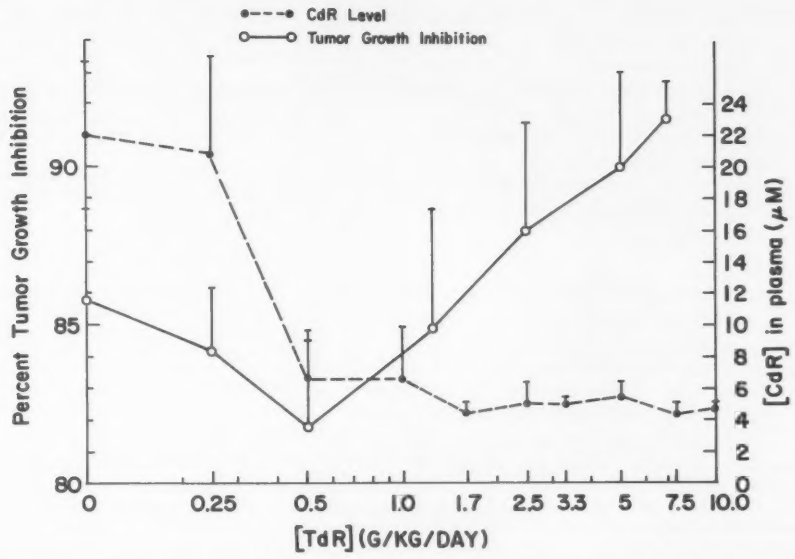

FIGURE 5.—Effects of TdR given by continuous infusion through the tail vein on the growth of rat colon tumors as affected by ara-C and on CdR levels in plasma. TdR at the doses shown was infused for 24 hr followed by 48 hr continuous infusion of ara-C alone at 175 mg/kg/day. Control animals (with no prior TdR infusion) received 48 hr continuous infusion of ara-C at 225 mg/kg/day.

resulting in the augmentation of the effects of an antimetabolite, ara-C. The plasma reduction in CdR may reflect intracellular modulation of dCTP by TdR. Thus, by monitoring the biochemical and pharmacological alternations induced by TdR in host and tumor tissues as a function of treatment, it should be possible to design optimal regimens providing the least toxicity and the most antitumor activity.

These in vivo studies in animals are being extended to an in vitro evaluation of the potential role of normal metabolites in the modulation of the metabolism and intracellular retention of ara-CTP in target cells using human normal and tumor cells.

CONCLUSION

Studies have been carried out in vitro and in vivo to identify the critical biochemical parameters that may aid in the prediction of response to ara-C and F-pyrimidines.

In the case of ara-C, systemic pharmacokinetic considerations may not be critical since steady-state levels of intact drug can be maintained in patients by continuous infusion. Target cell determinants could be identified, however, that seem to be critical as they correlate with tumor response. It has not yet been demonstrated to what extent there may be a direct relationship between a critical plasma level of ara-C and the extent of metabolic damage in target cells produced by the presence and maintenance of a specific minimum amount of ara-CTP. The studies discussed here indicate that in AML patients with no prior chemotherapy, the intracellular retention of ara-CTP correlates with the duration of first remission. Hence, factors that may contribute to the selective modulation of ara-CTP pools in tumor cells may eventually contribute to the modification of clincial response to this agent. Studies in animals have indicated that thymidine and possibly other nucleosides could be used to increase the intracellular pools of ara-CTP and, possibly, its retention.

In the case of 5-FU, although the consequences of F-pyrimidine incorporation into RNA in L1210/0 and L1210/FU cells have not been elucidated, it was found that resistant cells maintain lower pools of FdUMP and show less incorporation into RNA. Under the conditions of in vivo potentiation of 5-FU incorporation into RNA by TdR, the response of mouse colon tumors 26 and 38 was not modified above that obtained with 5-FU alone. Furthermore, in comparing the intracellular FdUMP pools derived from 5-FU, FUR, and FUdR and in correlating them with the in vivo response, the size of these pools and retention of FdUMP appear to be equally critical deteminants of response to these agents.

As a whole, these investigations have shown that it may be possible, with certain anticancer drugs and in certain diseases, to identify specific sets of parameters

in target cells that seem to have a determining role in drug action. In fact, it was possible to demonstrate that changes in these determinants correlate with therapeutic response. It was also demonstrated that the administration of certain normal metabolites may alter the response to an unrelated antimetabolite through metabolic modulation in target cells or changes in drug metabolism, both ultimately leading to establishing conditions favorable to the action of antimetabolite.

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Modulation of 5-FU Action by Thymidine in Murine and Human Tumors[1]

Charles W. Young[2,*], Thomas M. Woodcock[2], and Daniel S. Martin[3]

SUMMARY

The biochemical basis of, and preclinical experience with, combined chemotherapy with thymidine and 5-FU are reviewed. Phase I trial and clinical pharmacologic evaluation of a monthly schedule of thymidine and 5-FU showed marked increase in the biologic activity of 5-FU. Both toxic and antitumor effects were observed. Myelosuppression was the dose-limiting side effect. The central nervous system toxicity of 5-FU appeared increased; gastrointestinal toxicity was not. The available data do not permit an assessment of whether the therapeutic index of 5-FU has been altered by combination with thymidine. The basis for the observed increase in potency of 5-FU appears to be a thymine-induced marked slowing in its catabolism that results in a prolonged plasma half-life for 5-FU and an increased cellular exposure to the drug.

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Cancers manifest biological heterogeneity in their invasive, metastatic, and natural history characteristics and in their response to therapy. Failure of heterogeneous cancer cell populations to show complete response to single agent therapy is commonly visualized as a complex phenomenon. Cell death occurs principally among cells containing the requisite kinetic and biochemical characteristics that are relevant to the cytotoxic effects of the agent used. Other cells, lacking these characteristics in a quantitative or qualitative sense, survive to become the predominant cellular species; thus the tumor has acquired resistance to the drug in question.

The established success of combination chemotherapy is believed to be due, in part, to its capacity to approach heterogeneous tumor cell populations by several agents having significantly different biochemical mechanisms of action. In this treatment strategy the increased likelihood of therapeutic success would be based upon statistical probabilities. If the individual cytotoxic agents produce nonoverlapping host toxicity, they are used in dosages that approximate full dosage when used individually. If resistance to each of the drugs occurs independently, ie, in a nonlinked fashion, the statistical likelihood that a considerable population of tumor cells will be simultaneously spontaneously resistant to all three or four cytotoxic agents is markedly reduced because it is the product of the individual resistance frequencies, thereby providing an increased therapeutic index.

The problem of tumor cell heterogeneity is also being approached by using designed biochemical modulation of antimetabolite action. This concept is based upon the proposition that the metabolic differences within heterogeneous populations of tumor cells may not be so great that they cannot be made more homogeneous by manipulation with appropriately selected metabolites and antimetabolites. In this strategy, the administered dosages of the modulating metabolites and antimetabolites are chosen to produce the desired metabolic change rather than to be the maximally tolerated dose of each individual drug.

As illustrated in table 1, this approach can be visualized as using metabolites or antimetabolites to modify a primary antimetabolite effector. Generally the primary effector will be one that has direct entry into a macromolecule or produces a direct inhibitory effect on the synthesis of macromolecules. This concept has been explored most extensively with 5-FU as the effector (1–4), but regimens using arabinosylcytosine (5–13), mercaptopurine, or thioguanine (14–17)[4] and

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[4]Also explored by Martin DS, Sawyer RC, and Cadman R. Unpublished observations.

TABLE 1.—Biochemical modulations of metabolites and antimetabolites

Modulator	Modulation	Pharmacologic result	Reference no.
Thymidine	↓5-FU degradation	↑5-FU→FUMP	1–4
Thymidine	↓dTTP, ↓dCTP	↑AraC→AraCTP	5–9
Thymidine	"Selective" ↓DNA synthesis	Protection of non-neopolastic cells	10–13
PALA	↓Orotate, ↓pyrimidine pool	↑FU→FUMP	2
Methotrexate, DON	↑PRPP	↑FU→FUMP	14–17
MMPR		↑6TG→6TGMP	
Glutaminase	↓Plasma + cellular glutamine	↑Biological activity DON, AT125	18

6-diazo-5-oxo-norleucine as effectors (18) have also been advocated.

THYMIDINE AND 5-FU: MURINE OBSERVATIONS

The use of thymidine as a metabolic modulator of 5-FU was advocated by Martin who hypothesized that it would at least initially prevent dTMP deprivation and bring out thereby the cytotoxic effects derived from incorporation of 5-FU into RNA (1). Observations of Martin, Spiegelman, and Sawyer demonstrated in mice that TdR produced a fourfold increase of incorporation of 5-FU into nuclear RNA of 5-FU-sensitive tumors; in gut and bone marrow the increase was only 1.5-fold providing a possible biochemical basis for an increase in therapeutic index (1,2). Thymidine prevented a 5-FU-induced reduction in synthesis of DNA in the early hours following treatment; however, after 6 hours, inhibition of tumor cell DNA synthesis began that could not be corrected by further thymidine administration (2). The combination of TdR and 5-FU markedly increased the biological activity of 5-FU in mice (1,2) and humans (3). Thymidine is reported to increase the therapeutic index of 5-FU in a few rodent tumor systems but failed to do so in a number of others (19–21). In the following paragraphs the possible metabolic interactions of TdR and 5-FU will be considered as a prelude to presentation of the pharmacologic and therapeutic results of a phase I clinical trial of the combination. A more detailed report on these clinical studies has been published recently (3).

THYMIDINE AND 5-FU: BIOCHEMICAL INTERACTION

5-FU is believed to enter cells by facilitated diffusion; the process appears to be so rapid that phosphorylation is considered to be the rate-limiting step in this drug's anabolic sequence. According to current understanding, quantitatively the most significant step in in vivo 5-FU anabolism is its phosphorylation to FUMP by orotate-phosphoribosyltransferase. Because of the relatively high K_m for 5-FU in this reaction (around 2×10^{-4}M) (22), it proceeds at significant rates only briefly following a rapid iv injection of 5-FU because of the rapid catabolism of the drug in vivo. Any maneuver that retards degradation of 5-FU and thus maintains high blood levels for a prolonged period should enhance the drug's phosphorylation by this route. Any procedure that raises endogenous orotate levels, eg, therapy with pyrazofurin or allopurinol, should decrease 5-FU phosphorylation and reduce its potency in vivo (23).

5-FU may also be phosphorylated by the uridine phosphorylase and uridine kinase sequence according to the following reactions:

$$(a)\ \text{5-FU} + \text{R-1-P} \rightleftarrows \text{FUR} + \text{Pi}$$
$$(b)\ \text{FUR} + \text{ATP} \longrightarrow \text{FUMP} + \text{ADP}$$

Although the kinase reaction proceeds even with low concentrations of FUR, this pathway does not appear to be quantitatively significant in vivo for 5-FU, possibly because of low physiologic levels of R-1-P.

Thymidine is rapidly cleaved to thymine (T) and deoxyribose-1-phosphate (dR-1-P) by thymidine phosphorylase. This activity is present in most tissues; human plasma can also carry out the reaction. Thymine is then degraded to dihydrothymine (TH_2) by the same enzyme that reduces 5-FU (and uracil) to FUH_2 (or UH_2). This is the inital, and probably rate-limiting step in the progressive degradation of U and T. Martin and Spiegleman have confirmed Heidelberger's original observation that thymidine and thymine inhibit degradation of radiolabeled 5-FU. The consequence of this in vivo would be expected to be a prolonged elevation of plasma 5-FU levels and enhanced phosphorylation to FUMP by the PRTase pathway.

The increased deoxyribose-1-phosphate concentrations generated by degradation of TdR could be expected to lead to the synthesis of FUdR by a reversal of the thymidine phosphorylase reaction. This would be expected to lead to enhanced formation of intracellular

FdUMP by thymidine kinase reactions. The FdUMP generated in this manner, and that generated by the kinase, ribonucleotide reductase-phosphatase sequence, should maintain effective inhibition of thymidylate synthesis from deoxyuridylate. This would have no immediate impact upon synthesis of DNA because of the excess dTTP generated by the sequential phosphorylation of thymidine by the same kinase pathway. It is well established in vivo and in vitro that TdR can prevent the cytotoxic effects of low toxic concentrations of FUdR.

THYMIDINE AND 5-FU: CLINICAL OBSERVATIONS

Clinical trials of thymidine and 5-FU were initiated at several centers on the basis of Martin's initial report of enhanced therapeutic activity in murine breast and colon tumor systems. At the Memorial Sloan-Kettering Cancer Center a monthly dosage schedule was employed wherein the TdR was given by intravenous infusion over 30 minutes; 5-FU was administered at the conclusion of the thymidine infusion.

Pharmacologic Studies

Thymidine pretreatment (15 gm) all but eliminated oxidation of 2-^{14}C-FU as assessed by measurement of $^{14}CO_2$ in expired air and of ^{14}C-labeled urinary pyrimidines and catabolities by high pressure liquid chromatography (HPLC). In the absence of TdR pretreatment 75% to 80% of the administered ^{14}C-labeled 5-FU could be accounted for in expired $^{14}CO_2$; 20% of the administered ^{14}C was found in the urine but only 5% was 5-FU. With TdR pretreatment 55% of the administered dose was found in the urine principally as labeled 5-FU. ^{14}C-FUdR was present but ^{14}C-FUR was not detected. Combining expired ^{14}C-CO_2 and ^{14}C-containing products in the urine, 95% of administered 5-FU could be accounted for in the absence of TdR; however, only 60% of the administered radioactive 5-FU could be accounted for in the first 24 hours following TdR + 5-FU administration. Although fecal excretion was not examined, the data are consistent with an enhanced tissue retention of 5-FU when it is given concomitantly with large doses of TdR.

The plasma clearance of thymidine and of thymine is a saturable system. In humans given 3 g, 7.5g, 15 g, and 45 g respectively of thymidine, the terminal $T_{½\beta}$ values were: thymidine; 7, 24, 52 and 98 minutes. Thymine clearance was consistently slower; corresponding $T_{½}$ values were 32, 308, and 331 minutes. After 45 g, TdR plasma T levels were still rising at 6 hours; a meaningful $T_{½}$ could not be calculated. As predicted, the degradation of administered 5-FU was markedly prolonged in the presence of thymidine. The respective $T_{½\beta}$ values for 5-FU following TdR at 0 gm, 3 gm, 7.5 gm, 15 gm, and 45 gm were: 6, 33, 135, 180, and 190 minutes.

As had been predicted, FUdR was formed in relation to the quantity of TdR administered. After 45 gm TdR administration, the magnitude of this conversion was such that plasma [FUdR] > plasma [FU] in the initial time points. Plasma TdR levels always exceeded those of plasma FUdR.

Comparison of Murine and Human Pharmacokinetics

Mice have a greater capacity for pyrimidine catabolism than do humans. Although the murine TdR and 5-FU dosages employed by Martin were greater than those used clinically on both a weight and a body surface area basis, because of rapid murine catabolism the CXT for 5-FU over the first 3 hours in humans exceeded that in mice. This observation is consistent with the murine capacity to tolerate a weekly dose, while in the clinical trial at these dosages therapy could only be given on a monthly basis.

Phase I Trial: Dose Toxicity Relationships and Therapeutic Effects

Consistent with the observed prolongation of plasma 5-FU concentrations, TdR at 7.5- and 15-gm doses significantly increased the myelosuppressive effects of 5-FU (table 2). Given in this manner TdR 15 gm and 5-FU 7.5 mg or 10 mg/kg/day × 2 days produced life-threatening toxicity. Although the marrow toxicity was markedly increased, gastrointestinal toxicity was not increased in comparable degree. Even those individuals with life-threatening hematopoietic depression did not experience serious diarrhea or mucositis. This is consistent with the prior clinical experience that high dosage intermittent 5-FU produces greater myelosuppressive than gastrointestinal toxicity.

Central nervous system toxicity was also observed. This was similar to the neurotoxicity reported with 5-FU, ie, cerebellar symptoms, nystagmus, and disequilibrium. In some patients lethargy and confusion were also present. Although this was most pronounced in elderly patients and in patients of low performance status, this complication may limit repeated and prolonged use of thymidine and 5-FU regimens in the present configuration.

Therapeutic effects were observed in the course of the phase I trial, indicating that antitumor activity was potentiated in addition to the drug's hematopoietic and CNS toxic effects (table 3). Although the phase I population consisted largely of patients who had had prior chemotherapy, and 70% had received prior 5-FU, the two partial remissions and six minor regressions in 5-FU-pretreated patients and the partial remission in stage IV drug-resistant diffuse lym-

TABLE 2.—Hematopoietic dose-toxicity effects in phase I trial of thymidine and 5-FU*

TdR/5-FU dose	Number of patients	Wbc nadir (cells × 10^3/mm^3) Median	Range	Day	Platelet nadir (cells × 10^3/mm^3) Median	Range	Day
15g TdR + 5 mg/kg 5-FU daily × 2	2	1.0	(0.1–1.0)	16	70	(80–90)	14
15g TdR + 10 mg/kg 5-FU daily × 2	4	0.6	(0.1–1.5)	17	10	(8–12)	16
15g TdR + 7.5 mg/kg 5-FU × 1	17	2.3	(0.5–7.2)	16	149	(57–250)	14
15g TdR + 10 mg/kg 5-FU × 1	9	2.9	(1.0–6.2)	17	150	(58–198)	14

*Adapted from (3).

TABLE 3.—Antitumor effect of TdR/5-FU therapy in a phase I trial*

Tumor type	Number of patients	PR†	MR†	SD or PROG†
Carcinoma				
Colon	21	2	6	13
Ovary	2	1	—	11
Other	7	—	—	7
Leukemia/ lymphoma				
Myeloma	1	—	1	—
Hodgkin's	1	—	—	1
ALL	1	—	1	—
AMMOL	1	—	1	—
NHL	3	1	1	1

*Adapted from (3)
†PR = partial remission; MR = minor regression; SD = stable disease; PROG = progression.

phocytic lymphoma involving marrow and peripheral blood strongly urge that the thymidine-5-FU concept deserves extension into a phase II clinical trial in non-5-FU pretreated patients.

OTHER CONSIDERATIONS

Since the initiation of the clinical trials of TdR and 5-FU, other laboratory and clinical observations suggest possible further evolutionary paths for development of this therapeutic concept. Many workers have observed that at mM concentration, TdR produces a cytokinetic arrest of cellular progression through S phase. In some comparisons, nonneoplastic cells were more completely blocked in this progression than were the neoplastic cell varieties chosen for experimental comparison. Lee and colleagues reported that nonneoplastic cells tolerated mM TdR in culture for 48–72 hours with a considerably smaller loss of clonogenic capacity than they observed with their neoplastic counterparts (10).

Clinical trials were initiated at several centers on the basis of Lee's report that prolonged administration of TdR produced regression of human neoplasms growing as xenografts in immunodeficient nude mice (25). In these clinical trials the production of cytokinetic arrest of both normal and neoplastic cells has been documented by cytofluorometric analysis in patients when plasma TdR concentrations are maintained at mM levels in excess of 48 hours by continuous iv infusion (12,13). The selectivity of this effect is not established, but in at least a few patients where both normal and neoplastic cells were compared, the effects on the normal marrow exceeded those on the neoplastic cells[5]. Further exploration of this relationship is indicated to assess whether or not selective cytokinetic protection of normal cells is possible while leaving neoplastic cells relatively exposed to the cytotoxic effects of antimetabolites on macromolecular synthesis.

Other antimetabolites also alter the biological activity of 5-FU. Methotrexate and methylmercaptopurine riboside (MMPR) enhance 5-FU phosphorylation by increasing intracellular pools of PPRP, which may be the rate limiting substrate in the phosphorylation of 5-FU by orotate PRTase. N-(phosphonacetyl)-L-aspartate (PALA) enhances phosphorylation of 5-FU presumably by reducing intracellular orotate through inhibition of the de novo pathway of pyrimidine biosynthesis at the level of aspartate transcarbamylase. Orotate, the normal substrate competes for the PRTase that phosphorylates 5-FU and has a much greater affinity for the enzyme than 5-FU ($K_{m\ orotate}$ = 5 μM, $K_{m^5\text{-}FU}$ = 200 μM). Although normal orotate pools are small, physiologic concentrations probably compete effectively with 5-FU and limit its phosphorylation par-

[5]Woodcock, TM, Hansen HF, and Andreeff M. Unpublished observations.

ticularly when PRPP levels are marginal. When intracellular orotate levels are increased by inhibition of orotidylate decarboxylase through administration of allopurinol or pyrazofurin the biologic activity of 5-FU is markedly reduced (23). Martin and colleagues (19) and Johnson et al (25) have reported that PALA increases the antitumor effects of 5-FU and, in some tumor systems, increases the therapeutic index as well.

Inherent in all of these proposals is the hope that the selectivity of the antimetabolites' cytotoxic effects will be increased and that that increased selectivity will be beneficial rather than deleterious to the therapeutic result. Programs that visibly lessen the therapeutic index are not extended into clinical trial, but an understanding of the pharmcacology and the pathophysiology of the toxic effects may lead to modifications that enhance the therapeutic result. In the CD8 murine breast tumor model, Martin and colleagues have observed that the addition of uridine to thymidine and 5-FU-containing regimens significantly lessens toxicity without markedly impairing the antitumor effect. Subsequently they have demonstrated that pretreatment with testosterone materially increases tolerance of 5-FU and 5-FU-containing regimens (20).

Although the progressive addition of multiple agents makes evaluation of the pharmacologic and therapeutic impact of each individual component increasingly difficult, it should not preclude the practice. The development of four and five drug protocols in which there is no evident metabolic interaction has permitted major advances in the treatment of leukemia, lymphoma, breast, and testicular cancers. There is no need to preclude multiagent protocols merely because the components are visibly metabolically interactive and the optimal dose and time relationships difficult to ascertain. The need to provide a coherent base of experimental data on which to construct sound biochemically interactive treatment regimens is a significant challenge that will test the technical skill, imagination, and industry of this generation of pharmacologists.

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Pharmacology of Cyclophosphamide and Metabolites[1]

Michael Colvin,[2],* and John Hilton[2]

Cyclophosphamide is an alkylating agent described by Arnold and Borseaux in 1958 (1). Because of its wide spectrum of activity against human tumors and its high therapeutic index, the drug has become the most frequently used alkylating agent in cancer chemotherapy. Interestingly, the compound has been found to exhibit biological properties and toxicities that are not common to other alkylating agents. Since the parent compound is inactive in vitro, the biological effects of cyclophosphamide must be ascribed to metabolites. As the complex metabolism of cyclophosphamide has been elucidated, it has become possible to associate both the favorable therapeutic properties and the unusual biologic effects and toxicities with specific metabolites of the drug.

Cyclophosphamide is active against a wide range of animal tumors, and in most of these animal tumors it has a very high therapeutic index (2). That these characteristics are also exhibited in man is indicated by the broad range of human tumors against which the agents has shown a significant therapeutic effect and by the fact that these effects usually can be achieved at doses that do not produce severe toxicity.

In part, this high therapeutic index is related to a relative hematopoietic stem cell (3) and platelet sparing effect that is well-documented in animals and is also seen clinically. Prolonged depression of leucocyte levels after cyclophosphamide administration is rarely seen, even after high doses (4). Cumulative damage to the bone marrow, as manifested by decreasing tolerance to subsequent doses is also rarely, if ever, seen. With usual doses of cyclophosphamide, platelet depression is uncommon, and if seen in the absence of severe leukopenia, another cause should be suspected.

While many antitumor agents, including other alkylating agents, are immunosuppressive, the degree of immunosuppression produced by cylophosphamide is exceptional (5). Because of this effect, the agent has been used clinically in conditions where immunosuppression is indicated, such as renal and bone marrow transplantation and in the treatment of autoimmune diseases.

Two side-effects of cyclophosphamide that are uncommonly seen with other alkylating agents are alopecia and hemorrhagic cystitis. Alopecia is a frequent toxicity of other antitumor agents, including vincristine and adriamycin, but is usually not seen with alkylating agents. While the alopecia is not life-threatening and is always reversible, it is a distressing side-effect that is being seen more frequently as cyclophosphamide is used more frequently in combination with adriamycin. Sterile hemorrhage cystitis is essentially unique to cyclophosphamide and can result in severe hemorrhage and even death. The frequency of this complication can be reduced by careful attention to hydration of the patient and emptying of the bladder. Also, it has been demonstrated in both animals and man that either intravesicular or systemic administration of sulfhydryl compounds following cyclophosphamide can prevent hemorrhagic cystitis (6–8).

METABOLISM OF CYCLOPHOSPHAMIDE

The unique biological properties of cyclophosphamide are mediated by the compounds generated by the metabolism of the drug. The parent compound is not an alkylating or cytotoxic agent in vitro, but is activated by hepatic microsomal enzymes to a variety of active chemicals that are responsible for its biological effects. In the last 7 years the nature of the complex metabolism of the drug has been elucidated, and the generally accepted metabolic scheme is shown in figure 1 (9). The initial activation step is the oxidation of the heterocyclic ring to produce 4-hydroxycyclophosphamide. This compound is a hemiaminal that is in spontaneous equilibrium with an open-ring aldehyde, aldophosphamide. Spontaneous elimination of acrolein from aldophosphamide yields phosphoramide mustard, an active alkylating agent.

Enzymatic oxidation of 4-hydroxycyclophosphamide and aldophosphamide produces 4-ketocyclophosphamide and carboxyphosphamide, respectively. 4-Ketocyclophosphamide and carboxyphosphamide have no antitumor activity and little toxicity and appear to be detoxification products. These compounds, however, account for the majority of an administered dose of cyclophosphamide (85%), with carboxyphosphamide the major product and 4-ketocyclophosphamide the minor product. Nornitrogen mustard was originally postulated to be the alkylating agent produced by the

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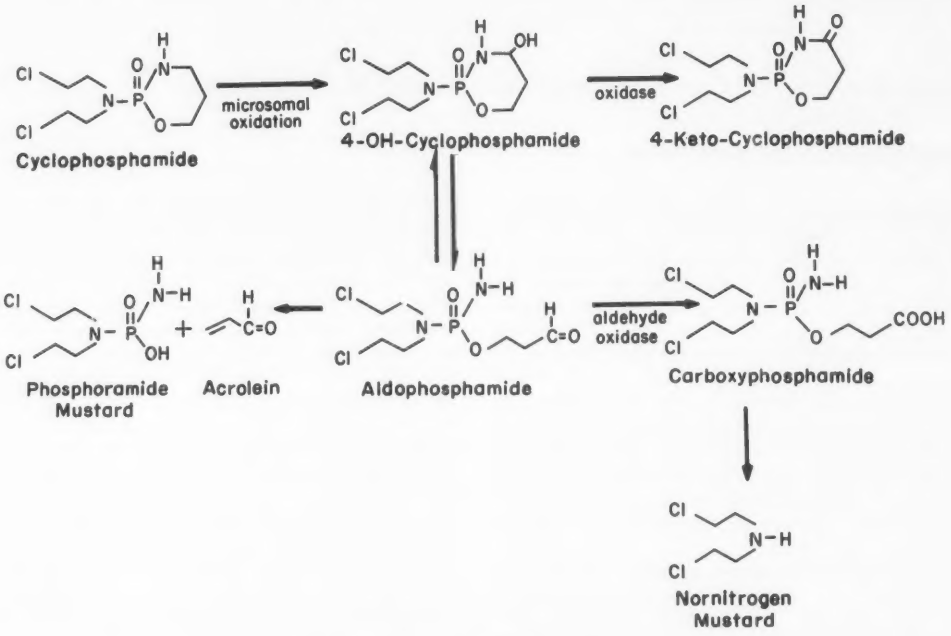

FIGURE 1.—Metabolism of cyclophosphamide.

metabolism of cyclophosphamide. Since this compound is an active alkylating agent and exhibits cytotoxicity both in vivo and in vitro, but exhibits little antitumor effect, it may play a role in the hematopoietic and other toxicities of cyclophosphamide. While postulated originally to arise principally from phosphoramide mustard, recent studies in our laboratory have indicated that this product is produced principally from the decomposition of carboxyphosphamide.

Acrolein, produced by the decomposition of aldophosphamide, is a toxic compound, and it has been suggested that it may be responsible for the antitumor effect of cyclophosphamide (10). However, at least two lines of evidence indicate that this compound does not play a role in the antitumor effect of cyclophosphamide. First, the levels required for cytotoxicity are considerably higher than those achieved after cyclophosphamide administration (11). Second, dechlorocyclophosphamide produces acrolein as readily as cyclophosphamide (12), yet has no antitumor activity. Recent data, however, suggest that acrolein may play a role in the toxicities of cyclophosphamide. Gurtoo has shown that acrolein released from 4-hydroxycyclophosphamide reacts with and denatures cytochrome P-450, both in vitro and in vivo (13). This effect may explain not only the inhibition of drug metabolism, which has been reported to be produced by high doses of cyclophosphamide, but also such unusual toxicities of the parent compound as the cardiac and pulmonary toxicities that can be seen with high doses of cyclophosphamide. Furthermore, Gurtoo has shown that the P-450 denaturation can be prevented both in vitro and in vivo by cysteine administration, suggesting that it might be possible to prevent some of the metabolic toxicities of cyclophosphamide by that means.

Also, both Cox (14) and Brock (15) recently have presented evidence that acrolein is responsible for the cystitis and renal damage that is produced by cyclophosphamide. However, in a phase I trial of phosphoramide mustard, hemorrhagic cystitis was observed in 8 of 40 patients,[1] indicating that phosphoramide mustard or its decomposition products play some role in this toxicity. Whatever metabolites are responsible for the bladder damage, a number of reports (eg, 6–8) have now shown that this damage can be prevented by the administration of either intravesicular or systemic sulfhydryl compounds.

Both phosphoramide mustard and 4-hydroxycyclophosphamide are cytotoxic in vitro and show a

[1] A. Skarin and E. Frei. Personal communication.

significant effect against animal tumors in vitro (16,17). Several authors have reported that 4-hydroxycyclophosphamide is more cytotoxic on a molar basis than phosphoramide mustard. Brock reported that 4-hydroxycyclophosphamide is 25 times more cytotoxic to Yoshida sarcoma cells in vitro than is phosphoramide mustard (18). In a recent study, shown in figure 2, Hilton has shown that 4-hydroxycyclophosphamide is cytotoxic to murine L1210 cells in vitro at one-fourth the levels required for toxicity by phosphoramide mustard. Correspondingly, when DNA crosslinking was measured by the alkaline elution technique, 4-hydroxycyclophosphamide was found to crosslink DNA at one-fourth the levels required for crosslinking by phosphoramide mustard (fig 3). Cytotoxicity with 4-hydroxycyclophosphamide and phosphoramide mustard was seen at equal degrees of DNA crosslinking. Furthermore, when DNA crosslinking in bone marrow cells and leukemia cells in vivo was studied, cyclophosphamide produced greater crosslink-

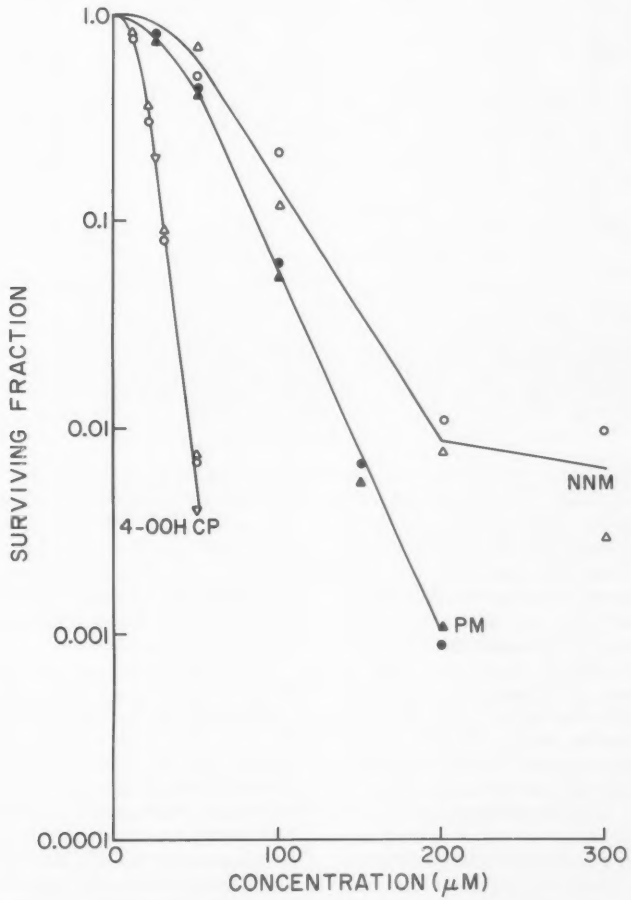

FIGURE 2.—Surviving fraction of L1210 cells exposed for 30 min to 4-hydroperoxycyclophosphamide (4-OOHCP), phosphoramide mustard (PM) and nornitrogen mustard (NNM). Each point represents the mean surviving fraction of triplicate samples from the same culture. Symbols indicate separate survival experiments. In our experiments the in vitro cytotoxicity of 4-OOHCP has been identical to that of 4-hydroxycyclophosphamide.

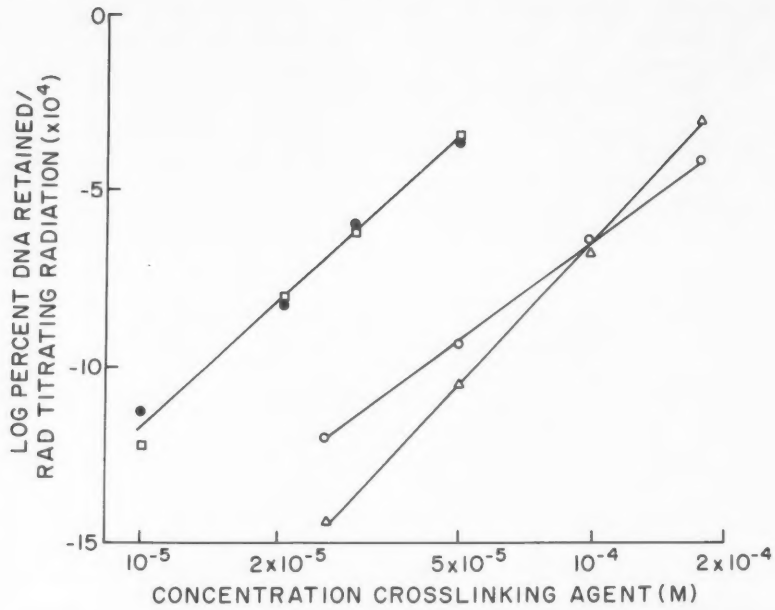

FIGURE 3.—Influence of various agents on the alkaline elution of DNA from L1210 cells exposed in vitro. Cells were exposed to the agents for 30 min at 37°, washed twice and returned to 37° in drug-free medium for 3.5 hr. The ordinate, log % DNA retained/rad titrating radiation ($\times 10^4$), is the slope of the radiation titration curve obtained from alkaline elution analysis. • = 4-hydroperoxycyclophosphamide; □ = 4-hydroxycyclophosphamide; o = nornitrogen mustard; △ = phosphoramide mustard.

ing in the leukemia cells than in the bone marrow cells (fig 4). This is in contrast to the results with phosphoramide mustard, which produced similar degrees of DNA crosslinking in the leukemia and bone marrow cells. These data are consistent with the hypothesis that 4-hydroxycyclophosphamide, which is not an active alkylating agent, exerts its cytotoxic activity through the crosslinking of DNA by phosphoramide mustard and can serve as a "transport form" of phosphoramide mustard. Thus phosphoramide mustard may enter the cell directly or in the form of 4-hydroxycyclophosphamide. Since 4-hydroxycyclophosphamide is cytotoxic at lower concentrations than phosphoramide mustard, this compound may enter cells more readily than phosphoramide mustard.

From the above data we conclude that most of the chemotherapeutic and cytotoxic effects of cyclophosphamide are due to 4-hydroxycyclophosphamide and phosphoramide mustard. The relative roles of these compounds will of course depend upon the concentration of each of them achieved in vivo and their respective biological properties. In turn, since at least the DNA damage by these two agents is due to phosphoramide mustard, any differing cellular effects of the two compounds are likely to be due to differing rates of cell uptake or differences in intracellular localization and metabolism. It has been postulated that normal cells may be able to oxidize 4-hydroxycyclophosphamide to 4-ketocyclophosphamide and carboxyclophosphamide more efficiently than tumor cells (11,19), but convincing evidence for this hypothesis has not been demonstrated.

In the animal test systems that have been studied, phosphoramide mustard has a much lower therapeutic index than cyclophosphamide. This has been shown by Brock in the Yoshida sarcoma (18) and in our experience with the L1210 murine leukemia. As shown in table 1, cyclophosphamide produces prolonged survival and some cures at an approximately LD_{10} dose. However, iv phosphoramide mustard produces less prolongation of life at doses that are more toxic. The two human trials of phosphoramide mustard (16,20) that have been carried out suggested considerable toxicity. Few antitumor responses were seen, although it must be considered that the patients had advanced disease and were heavily pretreated.

These observations indicate that the parent compound has a higher therapeutic index than phos-

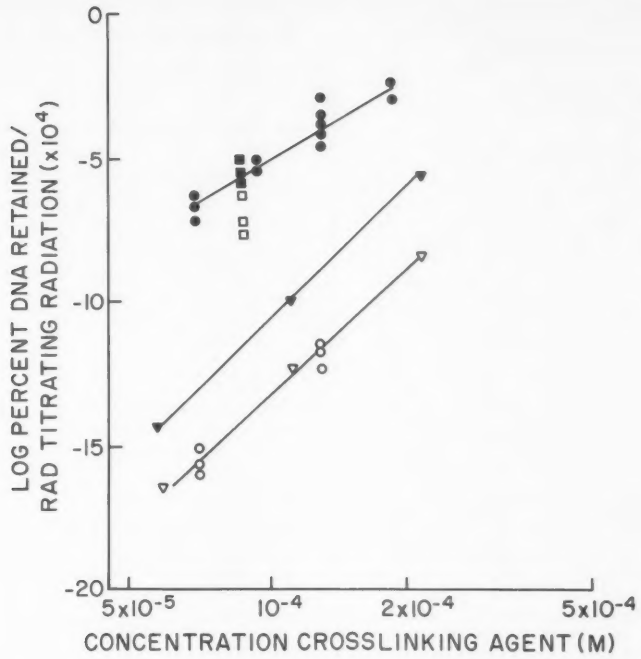

FIGURE 4.—Tumor-bearing mice received 100 μCi ^{3}H-TdR (7 Ci/mmol) ip 24 and 16 hr before iv administration of drugs. Tumor and marrow were harvested 4 hr after drug treatment for alkaline elution analysis. Each point represents material from one animal; filled symbols = L1210 cells; open symbols = marrow cells (o = cyclophosphamide; □ = phosphoramide mustard; ▽ = nornitrogen mustard.

TABLE 1.—Effect of intravenous drug on L1210 murine leukemia in vivo*

Drug	ILS†
Cyclophosphamide	
0.75 mmol/kg	73%
Phosphoramide mustard	
0.50 mmol/kg	37%
0.375 mmol/kg	94%

*Animals treated iv 4 days after injection of 10^5 L1210 cells ip.
†ILS = increase in life span.

phoramide mustard. From the nature of the metabolic scheme and the evidence presented above, we conclude that this higher therapeutic index is mediated by 4-hydroxycyclophosphamide.

Sensenbrenner et al have compared the relative effects of cyclophosphamide and phosphoramide mustard on hematopoietic spleen colony-forming cells and antibody-forming cells in mice (21). As shown in figure 5, a dose of 0.154 mmol/kg of phosphoramide mustard produced significant depression of hematopoietic cells, but there was little effect on the antibody-forming cells. In contrast, at the same dose of cyclophosphamide there was marked depression of antibody formation, but only a modest effect on the hematopoietic colonies. This work indicates that the immunosuppressive effect of cyclophosphamide is also mediated by 4-hydroxycyclophosphamide.

4-Hydroxcylophosphamide appears also to be responsible for the alopecia produced by cyclophosphamide. Feil and Lamoureux (22) found that phosphoramide mustard did not exhibit defleecing activity in sheep, in contrast to cyclophosphamide. Also, in the clinical trials of phosphoramide mustard (16,20) alopecia was not seen.

Very little is known about the clinical pharmacology of specific metabolites of cyclophosphamide. Levels of phosphoramide mustard have been measured by Jardine et al (23) in patients receiving 60 and 75 mg/kg of cyclophosphamide, and peak levels of 50 to 100 nmol/ml were found. As shown in figure 6, the levels of phosphoramide mustard were sustained for several hours. Maddock et al found inhibition of the growth of

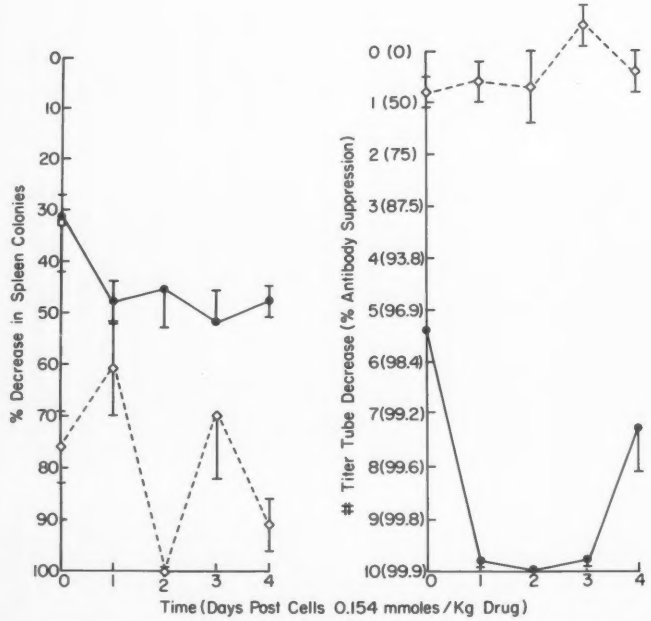

FIGURE 5.—Effect of 0.154 μmol of cyclophosphamide (o——o) and phosphoramide mustard (△——△) on spleen colonies expressed as mean percent decrease from untreated controls for at least ten mice at each data point (± SEM) and on SRBC titers expressed as the difference between the mean (± SEM) serial twofold dilution hemagglutinin titer of at least ten test animals and the mean titer of at least ten untreated control mice.

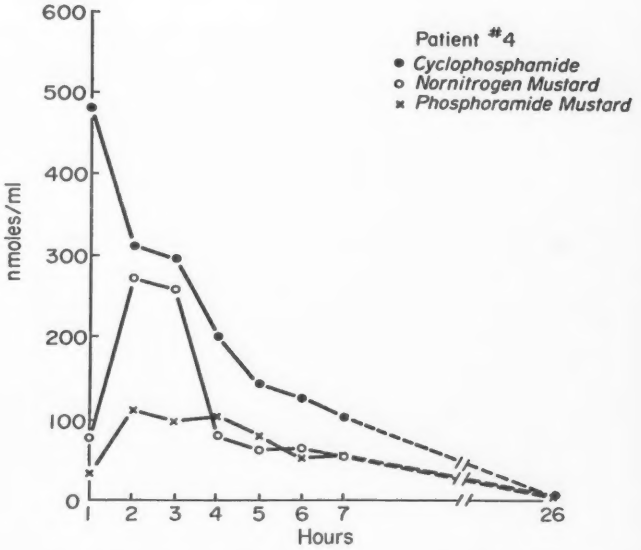

FIGURE 6.—Plasma levels (nmol/ml) of cyclophosphamide, nornitrogen mustard, and phosphoramide mustard for patient 4 determined from the end of a 1-hr cyclophosphamide infusion to 26 hr after the start of infusion.

cultured mamalian cells at concentrations of phosphoramide mustard in the range of 30–80 nmol/ml (16). Thus, at least after high doses of cyclophosphamide, toxic levels of phosphoramide mustard are found in human plasma.

Plasma levels of 4-hydroxycyclophosphamide have not been quantitatively measured in man. However, 4-hydroxycyclophosphamide has been detected in the plasma of patients after cyclophosphamide therapy by Fenselau et al as aldophosphamide cyanohydrin (24) and by Hohorst et al as a 4-thiol derivative of cyclophosphamide (25). In view of the greater cytotoxic effects of 4-hydroxycyclophosphamide it seems likely that cytotoxic levels of this compound do circulate.

CONCLUSION

In summary, the nature of the complex metabolism of cyclophosphamide has been elucidated in the past few years. The majority of an administered dose of cyclophosphamide is excreted as the inactive products carboxyphosphamide and 4-ketocyclophosphamide. Two toxic materials, acrolein and nornitrogen mustard, may play roles in the toxicities of cyclophosphamide. The therapeutic effects appears to be mediated ultimately by the alkylating and crosslinking agent phosphoramide mustard. This compound may enter the target cell either directly or as the nonalkylating precursor compound, 4-hydroxycyclophosphamide. The nonpolar 4-hydroxycyclophosphamide would be expected to enter cells more easily than the polar phosphoramide mustard. In particular, the high therapeutic index, the hematopoietic stem cell-sparing effect, and the immunosuppressive effect appear to be functions of either the cellular penetrative characteristics or the intracellular metabolism of 4-hydroxycyclophosphamide, since phosphoramide mustard does not display these properties. The relative roles of these two active metabolities will be more accurately defined as the relative levels of these compounds are measured in patients and as their relative biological properties are further determined.

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Clinical Pharmacology and Pharmacokinetics of cis-Platinum and Analogs[1]

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SUMMARY

cis-Platinum (DDP), the first metal coordination complex introduced into clinical trials, is remarkable for its therapeutic index. A short review of the numerator of this index, ie, the clinical activities of DDP given as a single agent or in combination therapy is presented. Toxicity of DDP, the denominator of the index, is given more attention, particularly nephrotoxicity, whose cumulative character and molecular mechanism are still in question and which can most often be prevented by following certain safety rules that are detailed in this paper. Pharmacokinetics data of free and filterable platinum are reviewed and discussed according to the different modalities of administration of DDP, and to what is known about its toxicity and its mechanism of cell kill. The rationale for using DDP in combination treatment is presented and the question of possible long-term toxicities is raised. *cis*-platinum analogs are sought for the purpose of enlarging the spectrum of activity, increasing selectivity and diminishing toxicity. Malonato-platinum has been shown not to be cross-resistant with DDP and to be clinically effective in adult acute leukemia. In a phase I study, malonato-platinum, which is poorly soluble, was administered in 6-24-hour infusions to 49 patients in doses ranging from 3 to 32 mg/kg. GI toxicity was universal. Hematological toxicity appeared to be mild and not clearly dose-related (the 3-32 mg/kg patients were not yet evaluable). Platinum pharmacokinetics in urine and plasma were performed using flameless absorption spectrophotometry. Preliminary results have suggested that malonato-platinum presented several pharp macokinetic features in common with DDP. Minor responses were seen in four solid tumor patients, three of whom were refractory to DDP. Other analogs soon to be introduced into clinical trials are listed.

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cis-Diamminedichloroplatinum or Cisplatin (DDP) is the first metal coordination complex, to be introduced into clinical trials. Discovered in 1965 by Rosenberg (1), it is one of the rare available oncostatic drugs powerful enough and with a high enough clinical therapeutic index to have radically changed the prognosis of some human malignant tumors, and has probably improved that of others at a reasonable toxic cost. The numerator of this index is the spectrum and the kinds of activities that DDP exerts on different tumors.

EFFECTIVENESS, TOXICITIES, AND CONTRAINDICATIONS

Table 1 shows the results obtained at Villejuif under the aegis of the French-American Agreement (2) and the overall benefical results mentioned in the literature (3,4) regarding DDP applied as a single drug. Applied in combination(s), DDP can cure metastatic testicular carcinoma of probably all cell types. For example, in Einhorn's study (5), there was an overall complete remission rate of 81% with 70% alive and 55% continuously disease-free from 21+ to 45+ months; 92% of these 55% have completed 2 years of chemotherapy and remain disease-free without treatment.

Using DDP in combination with adriamycin, vincristine, cyclophosphamide, 5-FU, and cytembena, we have obtained five complete remissions at surgical "second look" out of 11 patients with stages III and IV ovarian carcinoma. All of the complete remission pa-

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TABLE 1.—Activity of DDP as single agent in advanced malignancies

Tumor site	Source of data	Response rate (%)	
		Complete	Overall
Testis	French-American Agreement*	26	59
	Literature	19	60
Squamous cell carcinoma	French-American Agreement	7	46
Cervix	Literature	7	45
Head and neck	French-American Agreement	—	26
	Literature	6	28
Ovary	French-American Agreement	—	2/8
	Literature	15	32
Bladder	Literature	2	40
Prostate	Literature	—	43
Others			
Esophageal carcinoma			
Pediatric malignancies (osteogenic sarcoma, neuroblastoma)			
Lymphoma (Hodgkins and non-Hodgkins)			
Lung carcinoma			
Medullary carcinoma of the thyroid?			
CNS?			

*Studies at Villejuif by Hayat et al (2)

tients are still alive (6). Combined with adriamycin, vincristine, bleomycin, and cytembena, DDP induces an overall response rate of 60% in advanced squamous cell carcinoma from bronchus, head and neck, and cervix, and a major response rate (ie, complete and more than 50% regressions) of 40%, 40%, and 33% respectively (J.L. Misset, personal communication). These results may not be superior to the best series reported in the literature (7–10), but they are the best we have obtained with our own population of patients.

The denominator of the clinical therapeutic index is concerned with two problems: toxicities and contraindications, which are related. Table 2 lists these toxicities.

Nephrotoxicity has been studied extensively (11). The incidence and severity of platinum nephrotoxicity is dose-related. It appears that a single dose of less than 50 mg/m² usually will not be toxic, whereas 50–75 mg/m² will produce renal toxicity in a fourth to a third of the patients treated; the same toxicity is expected with a regimen of 15–20 mg/m² per day for 5 days. Nephrotoxicity, as reflected by increases in BUN and/or blood creatine to above normal limits is noted during the second week after cisplatin therapy and is mostly reversible in 3 or 4 weeks or more. At higher dosage, nephrotoxicity appears more frequently and is more severe. Moreover, it seems that in a significant percentage of cases it may be irreversible.

Light and electron microscopy studies of platinum-induced renal injury revealed tubular necrosis involving both proximal and distal convoluted tubules (12). Various methods have been used to alleviate cisplatin induced nephrotoxicity, including slow iv infusion of the drug, iv saline hydration with or without furosemide, and/or mannitol diuresis. There is no doubt that hydration with saline solution can significantly ameliorate or even prevent nephrotoxicity, but the gain due to mannitol or furosemide or both is still controversial (13,14). Cumulative nephrotoxicity has been indicated but cannot be ascertained unequivocally from the clinical studies performed. This point, however, is

TABLE 2.—Toxicities associated with DDP

Nephrotoxicity
Dose-related
Decreased (prevented?) by:
hyperhydration with saline solution
and furosemide (?) and/or mannitol (?)
Cumulative (?)
Gastrointestinal toxicity
Universal nausea and vomiting
Poor efficiency of antiemetics
Reduction by CIVI
Ototoxicity
Tinnitus
Hearing loss in the high frequency range
Dose-related
Cumulative
Neurotoxicity
Rare
Peripheral neuropathy
Demyelinative type
Other toxicity
Myelosuppression
Anaphylactic-like reactions
Electrolyte disturbance: hypomagnesemia
Ophthalmologic toxicity (?)

important as many physicians will not exceed a 600 mg/m² total dose even if a therapeutic gain may still be expected. With A. Leroy from NIH, we studied sections from kidney biopsies by electron microscopy to detect ultrastructural changes in patients treated with large cumulative doses of platinum compounds, and confirmed previous reports of accumulation of electron-dense material within intracytoplasmic vacuoles (12). Using the electron probe microanalyzer, we are studying the elemental composition of these inclusions. In the two patients studied, we have found that if platinum is accumulated, it is below present detection level with the electron probe. We are taking steps to lower this detection limit.

In phase II studies (15–18), 100% of the patients treated with DDP at doses of 30-100 mg/m² experienced GI toxicity, corroborating the data of phase I studies (19–23). Nausea and vomiting usually begin 1-2 hours after administration and lasts 24-48 hours, sometimes much longer. Antiemetics are of little help, although the partial efficiency of prochlorperazine, chlorpromazine, and more recently droperidol (24) and nabilone (25) have been reported. Continuous iv infusion has been reported to significantly reduce the incidence as well as the intensity of GI toxicity (26,27). On the other hand, vomiting was as frequent and more prolonged when DDP was given in infusion compared to iv bolus injection in a randomized trial (28).

As far as ototoxicity is concerned, hearing loss in the high frequency range is dose-related and this toxicity appears cumulative. It is not clear if it can be reversed. Hydration and diuresis do not appear to lessen this toxicity, to which older patients are more sensitive (29). Neurotoxicity occurs much less frequently and consists of peripheral neuropathy, mainly sensory. This neuropathy has been shown to be of the demyelinative type (30).

Other toxicities are known: myelotoxicity is generally moderate and appears to be dose-related and possibly cumulative. Hydration and diuresis do not alter the incidence and severity of myelosuppression (29). Anaphylactic-like reactions have been described (31,32). Hypomagnesemia, most likely due to tubular damage, has been recently reported. In association with hypocalcemia, it may induce tetany in younger patients (33).

This long enumeration of toxicities must not lead to an overestimation of the therapeutic cost. Safe use may be expected if one uses the hyperhydration technique with or without mannitol diuresis and/or furosemide, takes into account the primary contraindication (ie, renal insufficiency), makes dose adjustment in case of structural or functional renal impairment (which is common in elderly patients), avoids association with macrolides that may increase nephrotoxicity (12), and carefuly monitors the patient, especially in clinical situations of third spacing (ascites, edema) (29). As an example, figure 1 shows the protocol for DDP as a single agent used at Villejuif under the French-American Agreement.

In conclusion, DDP is already one of the most effective oncostatics, and it is essential that its use be further improved by acquiring additional pharmacologic knowledge.

Is it possible to decrease the main immediate and sometimes long-lasting side effects, of nausea and vomiting, and thus treat the patients as outpatients? The answer is no, since nausea and vomiting are universal in dose above 20 mg/m². Fractional schemes are therefore useless, since even newly introduced antiemetics are poorly effective.

Is the infusion mode of administration more advantageous than bolus injection in the pharmacokinetics studies, and do hyperhydration and forced diuresis change the pharmacokinetics? Both questions are important since a wide variety of dose schedules has been employed, as shown in table 3, which is borrowed from Muggia, Rozencweig, and Penta (personal communica-

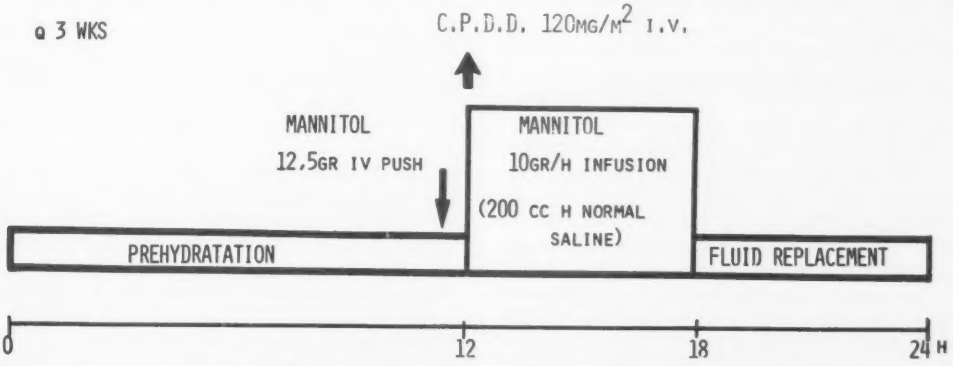

FIGURE 1.—Protocol of treatment with DDP, single drug.

TABLE 3.—Mode of administration of cisplatin in protocol studies*

Protocol	Total dose per course (Mg/m^2)	Schedule	Infusion duration	Mannitol	Furosemide
CI	70	d 1 q 3 wks	1 hr	+ Inf†	+
ALGB 260	50	d 1 q 3 wks	1-2 hr	+	0
ALGB 0703	70	d 1 q 3 wks	Bolus	+	0
CSG 071	75	d 1q 3 wks	8 hr	+	+
SWOG 7730	100	d 1 q 3 wks	Bolus	0 vs +	0
SWOG 7611	75	d 1-5 q 4 wks	Bolus	0	0
SWOG 7629	100	d 1,8 q 4 wks	Bolus	+	0
ALGB 7771	120	d 1,2 q 3 wks	4 hr	+ Inf†	(+)§
Roswell (Merrin)	60	d 1 q wk	6 hr	+ Inf†	0
Stanford (Jacobs)	80	d 1 q 3 wks	24 hr	0	0

*From Muggia et al (personal communication).
†Inf = infusion of mannitol spanning DDP administration.
§( ) = Specified only if urinary output © 50 ml/hr was found during mannitol administration.

tion). In any case, there is no strong clinical evidence for the superiority of one schedule over the other. It must be recalled that in L1210 murine leukemia, DDP activity does not display appreciable schedule dependence. Its toxicity has been attributed to interactions with DNA that are similar to the interactions of bifunctional alkylating agents. DDP seems to be a cell cycle-nonspecific agent (34).

PHARMACOKINETIC STUDIES

Initial pharmacokinetic studies in man involved use of radioactive DDP. It was shown by DeConti in 1973 (19) that levels of radioactivity decay in a biphasic manner with a T 1/2α of 25-49 minutes and a T 1/2β of 58-73 hours. During the excretion phase, over 90% of the radioactiviity in the blood is protein-bound. Urine excretion is incomplete and prolonged. In the same period Lange (35), using radioactive DDP in two patients and whole-body scanning, was able to demonstrate that after 3 hours radioactivity was concentrated in the kidney and in the head regions, but not in the brain. After 40 hours, organs containing most of the radioactivity were the kidney, liver and intestine. The patterns of clearance of cisplatin from plasma were similar to those described previously.

More recently, flameless atomic absorption spectrophotometry (AAS) (36) and centrifugal ultrafiltration procedures (37) have enabled measurements of the free or filterable drug and protein-bound or nonfilterable drug. Thus current studies deal with the behavior of free and total platinum species. For example, Gormley et al. (38) have shown that for a dose of 70 mg/m^2, the mean peak plasma level is 30 μM. The decay is biphasic for both free and total platinum. For total platinum the T 1/2α is 23 min, the T 1/2β is 67 hours. Renal excretion is about 23% at 24 hours. For free platinum the T 1/2α is 8-10 minutes, and the T 1/2β is 40-45 minutes, with a plasma concentration below 0.5 μM in 4 hours. Renal clearance of platinum indicates that free platinum is probably excreted primarily by glomerular filtration.

We have measured free and total platinum concentrations. Figure 2 shows the plasma decay of free platinum in five patients who received 120 mg/m^2 of DDP with hydration and mannitol diuresis. The mean peak plasma level was 18.7 μM $\pm$5.7 and the mean T 1/2β was 32 minutes. We are now studying DDP kinetics when the drug is given alone or in combination chemotherapy.

In Belt's study (39), dealing with free platinum only, peak plasma levels were lower, elimination half-time shorter, and excretion greater with infusion than with bolus. In both administration methods, mannitol increased the peak plasma level and markedly decreased urinary elimination. Thus with infusion, as opposed to bolus administration, peaks are lower and excretion becomes proportionally greater as nonprotein bound platinum is continuously and efficiently excreted. Surprisingly, mannitol exerts an effect favoring retention of platinum. Recent studies reported by Ostrow (40), when compared to Belt's studies, imply that a similar effect on renal excretion may be observed after furosemide. These results contradict those reported by Simone (41) in the rat, where mannitol, but not furosemide, diuresis caused an increased urinary excretion.

The non-protein-bound species is likely to be the active one. This has been suggested by Patton in studies using human bone marrow colony-forming units in culture (42), and Gormley using L1210 mouse leukemia cells (38). Hence we are facing the dilemma of whether to use infusion instead of bolus to decrease nausea and vomiting and renal toxicity, if these are related to peak plasma levels, or to use bolus injection to get higher drug levels and longer excretion half-lives, especially since Drewinco has shown that the cell kill of human

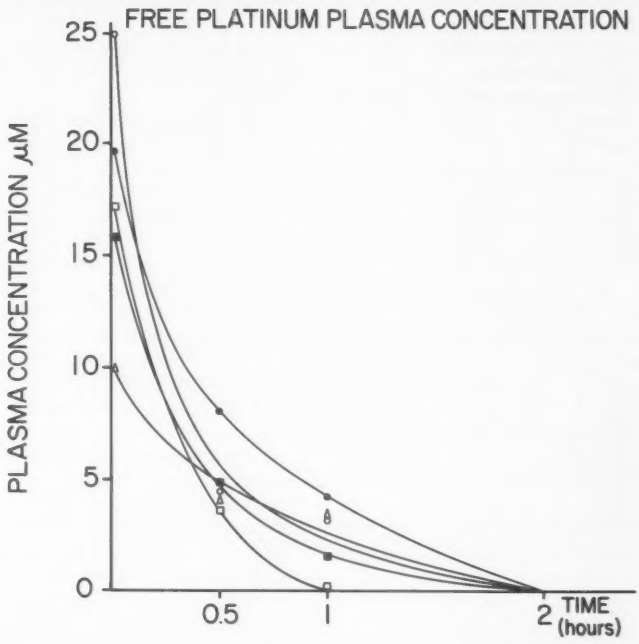

FIGURE 2.—Plasma free platinum in five patients who received 120 mg/m² of DDP with hydration and mannitol diuresis.

lymphoma cells by DDP is influenced by dose and duration of exposure (43).

Lastly, mannitol, which may have a protective effect on the kidney, also seems to be advantageous in increasing terminal half-life of free platinum, by an unknown mechanism.

COMBINATION WITH OTHER DRUGS

What is the rationale for DDP combinations, which have been shown in numerous studies to be more effective than DDP given alone? First, because DDP may act as an alkylating agent, it is logical to combine it with drugs acting by other mechanisms. Second, combinations with cyclophosphamide, ifosfamide, thioguanine, methotrexate, or daunomycin enhanced the therapeutic effects of DDP when compared with either drug used alone in the treatment of L1210 leukemia (4). Third, its very particular toxicity makes combinations with other more myelotoxic drugs easier. Fourth, DDP has been shown by Rosenberg to bind to cell membrane nucleic acids and possibly make the immune mechanism contribute to its antitumor activity (44). Thus it may be of interest to combine it with immunoadjuvants.

The question of possible long-term toxicities of the agent is important since DDP is being used in adjuvant chemotherapy trials for poor prognosis cancer stage I and for stage II testicular cancer at the Memorial Sloan Kettering Cancer Center (45) and in our group. Mutagenicity is a known effect. Long-term renal effects have not yet been studied with the new administration modalities. In the case of testicular cancers, this makes the "wait and see" attitude of Einhorn very attractive (46).

MALONATO-PLATINUM AND OTHER ANALOGS

Cisplatin analogs are sought to enlarge the spectrum of activity, increase selectivity, and diminish toxicity. Since there are tumors that show primary or secondary resistance to DDP, we were interested in the demonstration by Burchenal that malonato (1,2-diamminecyclohexane) platinum (malonato-platinum) does not show cross-resistance with DDP (47). Moreover, Hill et al had published impressive results with this derivative, especially in patients with acute myeloid leukemia (48). We were aware that they had administered doses as high as 16 mg/kg.

We therefore conducted a preclinical toxicological study of this agent in baboons (49). The main parameters we followed were fever, blood pressure, urine volume, and behavior, as well as renal and liver function. We gave the first baboon 16 mg/kg of malonato-platinum, dissolved in water with 5% glucose, 10% propylene glycol, and 0.2% sodium chloride by heating and magnetic stirring. One day later the baboon was listless, and died on the fifth day. Renal failure was observed, and necropsy showed tubular necrosis. We then tried smaller doses (6 and 10 mg/kg) and saw no toxicity. We could eliminate direct toxicity of the diluent. In the last study we injected 16 mg/kg dissolved in water with 5% glucose. The preparation of this solution was quite difficult because of poor solubility and required the help of a sonicator. There was no toxicity. We concluded that malonato-platinum is not toxic per se at this dose but in the first experiment had been converted by heating into another compound very toxic for the kidney.

In a phase I trial (49), we studied 49 patients with doses ranging from 3 to 32 mg/kg (table 4). The methods of administration were the same as for DDP, ie, fluid loading at the lower dosage, mannitol diuresis, and fluid replacement. As far as nonhematologic toxicity is concerned, we observed GI toxicity: nausea was universal, vomiting frequent, and diarrhea occasional. No renal toxicity was seen, as reflected by BUN and creatinine values, nor was there any neurotoxicity or ototoxicity. Blood magnesium was not checked. Hematologic toxicity appeared to be mild and not clearly dose-related. However, at the 24-mg/kg dose level, moderate thrombopenia may be expected 15-21 days after treatment with complete recovery by Day 28. Hematologic toxicity did not seem to be cumulative (table 5).

TABLE 4.—Phase I trial of malonato-platinum in 49 patients

Doses (mg/kg)	Number of patients
3	1
6	1
8	1
10	15
16	17
20	4
24	7
32	3

The platinum pharmacokinetics in urine and plasma from eight patients were studied with Drs. N. Alcock and D.P. Kelsen using flameless AAS. Dose levels ranged from 3 to 24 mg/kg.

Peak concentrations ranged from 1.1 μg/ml to 20.5 μg/ml. The excretion phase had a mean T 1/2β value of 63.5 hours (range 42–92 hours). Figure 3 demonstrates the plasma platinum concentrations at the 16 mg/kg dosage level during the first 24 hours. Urinary excretion was measured in one patient receiving 16 mg/kg. In 4 days this patient eliminated 37.5% of the total administered dose. We are presently studying free and total platinum plasma kinetics at the highest dosage level.

Of the 44 patients who received 10 or 24 mg/kg per course and had a measurable disease, 36 are evaluable (table 6). The results are as follows (table 7). No response was seen in patients with acute leukemia. Responses were observed in only four patients with solid tumors. Three patients received 1 to 3 cycles. For one the dose was 10 mg/kg, for two it was 16 mg. One patient had received no previous chemotherapy and was given 2 x 20 mg/kg courses. In a patient with an ovarian carcinoma, the result was alleviation of intestinal obstruction. In a patient with a mediastinal embryonal carcinoma, the evaluated parameter was a superior vena cava syndrome. A patient with a bronchus carcinoma (squamous cell type) experienced a decrease in nodular skin metastasis. In a patient with a testicular embryonal carcinoma, the result was pain suppression, normalization of hepatic tests previously altered by liver metastases, and a decrease of a lumboaortic tumor mass as shown by two consecutive CAT scans.

Other analogs such as *cis*-dichloro-*bis* (cyclopentylamine) platinum (II) (PAD), platinum uracil blues, sulfato (1,2)-diaminocyclohexane platinum (SHP), and carboxyphthalato-1,2-diaminocyclohexane platinum will

TABLE 5.—Hematologic toxicity of malonato-platinum in patients with solid tumors

Cell type	<10mg/kg /N° Courses	10mg/kg /N° Courses	16mg/kg /N° Courses	20mg/kg /N° Courses	24mg/kg* /N° Courses
PMN	1^{+++}	1^{++}	1^{++}		
	1/2	$2/8^{1}+$			
			1/15	0/7	0/9
Platelets	1^{+}	2^{+++}	2^{+}		5^{+}
	1/2	$4/7^{2+}$	2/15	0/7	5/9

PMN: (1,500–1,000)$^{+}$
(999– 500)$^{++}$
PMN: (< 500)$^{+++}$

Platelets: (100,000–50,000)$^{+}$
(49,000–20,000)$^{+++}$
(< 20,000)$^{+++}$

*The 3 patients treated with 32 mg/kg are not yet evaluable.

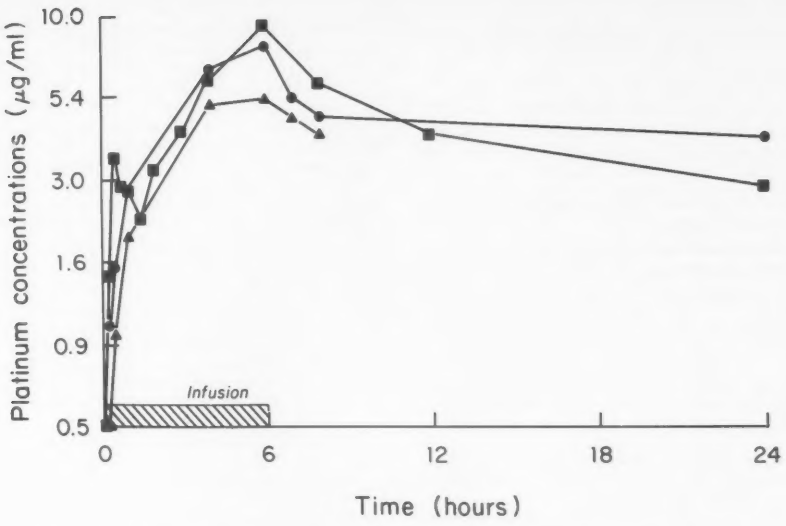

FIGURE 3.—Plasma platinum during and after a 6-hr infusion of malonato-platinum 16 mg/kg (three patients). (The 48-, 72- and 96-hour measurements are not shown.)

TABLE 6.—Characteristics of patients treated with malonato-platinum

Number of patients summitted to trial		44
Number of evaluable patients		36
Sex	Age	
Female, 15	Mean 39.5, range 13–55	
Male, 29	Mean 40, range 12–69	
Prior treatment		
Chemotherapy only		28
Radiation therapy only		1
Radiation therapy and chemotherapy		14
No prior treatment		1

TABLE 7.—Malonato-platinum results

Diagnosis	Total number	Responses					Resistance to DDP		
		CR	PR	MR	NC	PD	Yes	No	Uncertain
AML	3	—	—	—	—	3	—	3	—
ALL	1	—	—	—	—	1	—	1	—
Embryonal carcinoma	11	—	1	1	1	8	8	—	3
Ovary carcinoma	3	—	—	1	—	2	3	—	—
Squamous cell									
Lung	6	—	—	1	—	5	—	6	—
Cervix uterus	2	—	—	—	—	2	1	—	1
Miscellaneous	10	—	—	—	—	10	—	10	—
Total	36	—	1	3	1	31	12	20	4

CR = Complete response PR = Partial response MR = Minor response
NC = No change PD = Progressive Disease

be introduced into clinical trials. As Muggia has pointed out (personal communcation), the major features of interest of these analogs, other than their antitumor activity, should be the absence of cross-resistance to DDP, solubility and stability, reduced nephrotoxicity, lipophylicity, and penetration into the CNS.

In conclusion, the platinum complexes have not only radically changed the prognosis of some human tumors and encouraged research on the possible absence of cross-resistance between analogs, they may also have opened the door to new sources of oncostatics, namely metals.

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Ellipticine, 9-Hydroxyellipticine, and 9-Hydroxyellipticinium: Some Biochemical Properties of Possible Pharmacologic Significance[1]

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SUMMARY

Ellipticine, 5,11-dimethyl-(6H)-pyrido-(4,3,b)-carbazole (NSC 71795) is a potent cytotoxic and antitumor agent. Several of its derivatives share these properties and nine hydroxylated compounds are even more potent. 2-Methyl-9-hydroxyellipticinium (NSC 264-137) has thus recently been shown to display some therapeutic effectiveness in human breast cancers. The biochemical mechanism of this action is discussed in this paper. DNA has been implied as the target of ellipticine and some derivatives; however, these drugs do not break DNA in cultured L1210 cells. Moreover, several other biochemical constituents are able to bind these compounds in vitro as efficiently as DNA. In this paper the interactions between ellipticine and some of those derivatives and (*a*) ascitic phospholipids monolayers and (*b*) cytochrome P450 microsomal proteins are studied and discussed. Finally, the hypothesis that the nonhydroxylated ellipticine derivatives might be better cytotoxic agents because they can be oxidized and generate free radical species through a monovalent electron transfer process is presented. This hypothesis is supported by experiments carried out on the oxidation of 9-hydroxyellipticine and 2-methyl-9-hydroxyellipticine by peroxidases.

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Ellipticine, 5,11-dimethyl-(6H)-pyrido-(4,3,b)-carbazole (NSC 71795), and some of its derivatives (fig 1) are active antitumor agents in animals and humans; for example, 9-methoxyellipticine has been reported to induce remission of myeloblastic acute leukemia (1) whereas, more recently, 2-methyl-9-hydroxyellipticinium has been shown to give rise to partial remission in about 25% of treated cases of breast cancer no longer responsive to any other therapy (2,3, and EORTC preclinical screening group unpublished results). Those drugs very probably owe their anticancer action to their cytotoxic properties (4), but it is presently difficult to relate their effects to any precise biochemical mechanisms.

Ellipticine and derivatives are endowed with several chemical characteristics that might come into play during the vast array of biochemical and pharmacological actions of these drugs. The unsubstituted dimethylpyridocarbazole structure is hydrophobic and planar. Both these properties partly explain why and how ellipticine and most of its derivatives bind strongly to DNA: the apparent association constants of ellipticine with calf thymus DNA at a binding ratio of 1 ellipticine/20 nucleotides at pH 7.0, 0.1 M Na^+, 25 °C is 1 x 10^7 1/mol (5). Many compounds in this series intercalate between base pairs (6,7). The hydrophobicity of most of these drugs might also explain why ellipticine and 9-methoxyellipticine strongly interact with phospholipid monolayers (8,9). Another interesting feature of ellipticine is the existence of a pyridine nitrogen that confers on these molecules the possibility of interaction with electrophilic centers; together with the hydrophobicity, this kind of interaction provides a rational basis for understanding the strong affinity of ellipticine and some of its derivatives towards enzymes con-

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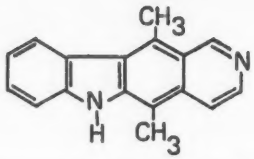
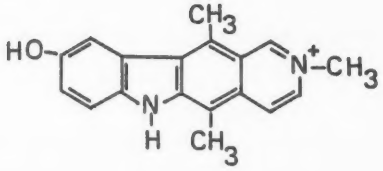
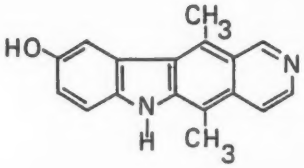


ELLIPTICINE (NSC 71795)



2-METHYL 9-HYDROXYELLIPTICINIUM
(NSC 264137)



9-HYDROXYELLIPTICINE
(NSC 210717)

FIGURE 1.—Structures of ellipticine, 9-hydroxyellipticine, and 2-methyl-9-hydroxyellipticine.

taining heme iron, such as peroxidase (10) and cytochrome P450 enzymes (11). Finally, the 9-hydroxylated ellipticine derivatives that are more cytotoxic than their nonhydroxylated analogs (4) might be envisioned as the reduced form of a quinone-imine structure; the oxidation of these hydroxylated compounds might be accompanied by the formation of highly cytotoxic free radicals.

The present report briefly documents and comments on the above biochemical properties of ellipticine, 9-hydroxyellipticine, and 2-methyl-9-hydroxyellipticinium and their possible pharmacologic significance.

EFFECT OF ELLIPTICINE AND SOME 9-HYDROXYLATED DERIVATIVES ON DNA IN VITRO AND IN VIVO

The characteristics of the in vitro interaction between DNA and ellipticine and other compounds in this series have been previously described and the binding constant determined (5,12). In vivo, ellipticine, like most of the clinically useful antitumor antibiotics, alters the functions and the synthesis of nucleic acids; it inhibits the incorporation of labeled thymidine into DNA of L1210 (13) as well of *Escherichia coli* cells (14). It is also mutagenic in *Salmonella* (15) and in yeast (M. Pinto and C. Paoletti, unpublished observations).

Alkaline gradient sedimentation reveals that DNA extracted from L1210 cells exposed to ellipticine is apparently fragmented (16) (fig 2). When monitored by an alkaline elution technique, this apparent fragmentation can also be observed but only after treatment of DNA in the lysis medium by proteinase K (17).

These results raise two major questions. Is this apparent fragmentation accompanied by a true breaking of some phosphodiester bonds of DNA inside the cells or is it induced by the in situ generation of alkaline sensitive regions either in DNA or in the nucleic acid-protein nuclear complexes? Does it happen in the same range of drug concentrations that alter the biological functions of the cells related to their survival and division? We describe in this paper some experiments and discuss some conclusions pertaining to these questions.

There is no experimental evidence of any occurrence of breaks in DNA inside the cells exposed to ellipticine and its two hydroxylated derivatives. This observation was made thanks to the very sensitive method first described by Cook and Brazell (18). Nuclear structures, called nucleoids, very much enriched in DNA-protein complexes, are released by gentle lysis in a neutral solution containing a nonionic detergent, 0.5% Triton X-100 in 1.95 M NaCl, 0.01 M pH 8.0 Tris, and 0.001 M EDTA and sedimented in a neutral sucrose gradient. When the nucleoids are extracted from cells exposed to some antitumor drugs, at some concentrations, they sediment at a reduced rate when compared to nucleoids from untreated cells. Moreover, the nucleoids sediment

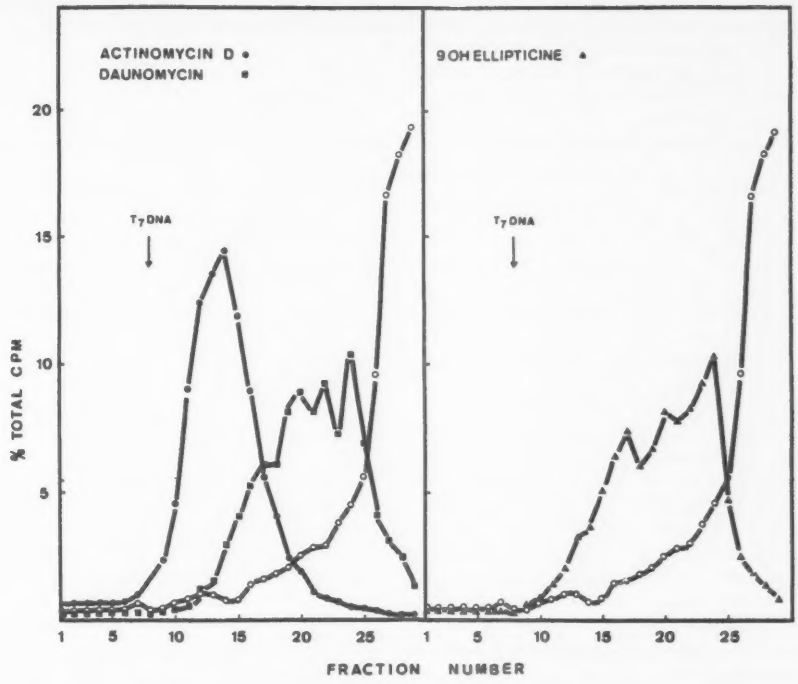


FIGURE 2.—Alkaline sucrose sedimentation of DNA extracted from in vitro grown L1210 cells exposed to 19-hydroxyellipticine. L1210 cells (2 x 10^5 cell/ml), grown as previously described (17), were prelabeled during 20 hr in presence of [methyl-^{14}C]-thymidine (0.06 μCi/ml). The cells were resuspended in fresh medium and exposed to 90-OH ellipticine (1000 ng/ml) for 15 min at 37 °C. Centrifuged cells were resuspended in NaCl 9%. 500 μl of suspension (7 x 10^4 cells) were lysed during 20 hr at 4 °C on top of linear alkaline sucrose gradient (5%–20%), 0.5 M NaCl, 0.3 M NaOH, 0.01 M EDTA. The lysis solution (800 μl) was made of Sarkosyl 1% in alkaline sucrose 2.5%. Centrifugation was for 270 min at 25 Krpm in Spinco SW27.1 rotor at 20 °C. Each fraction: 18 drops. Recovery: 85%. Intertechnique SL30 counting. ○ = controls with no drug; ● = actinomycin D, 10,000 ng/ml for 3 hr; ■ = daunomycin, 1000 ng/ml for 3 hr.

in a neutral sucrose gradient that contains the intercalating agent, ethidium bromide, in a manner characteristic of a supercoiled DNA. As the concentration of ethidium bromide in the gradient increases, the distance traveled by the DNA-protein complexes falls to a minimum for a dye concentration of about 4 μg/ml and then increases again; if the supercoiling of DNA that is wrapped in the nucleoids is lost as a consequence of the introduction of single-strand or double-strand breaks, this typical biphasic effect is not observed. Using this technique we were unable to observe the occurrence of any breaking of DNA in nucleoids extracted from L1210 cells treated with ellipticine or its two derivatives (fig 3). As a control, we monitored breaks in DNA originating from cells exposed to actinomycin D, which has been previously described as a true DNA breaking substance (19,20).

These data indicate that the decrease of the DNA sedimentation rate that occurs in the alkaline environment prevailing during cell lysis and sedimentation is not due to an ab initio breaking of DNA inside the cells exposed to ellipticine or its two hydroxylated derivatives. Two possible explanations can be suggested: (*a*) some alkaline labile regions might be generated in DNA of cells in the presence of ellipticine, 9-OH-ellipticine or 2-CH_3-9-OH ellipticine[6], for example, one might suppose that the covalent binding of 9-OH-ellipticine or 9-OH-ellipticinium, associated with the metabolic oxidation of these compounds (see below), might generate zones of fragility in the DNA structure, which would be subsequently revealed by the increase

[6]Abbreviations: 9-OH-ellipticine = 9-hydroxyellipticine 2-CH_3-9-OH-ellipticine = 2-methyl 9-hydroxyellipticine.

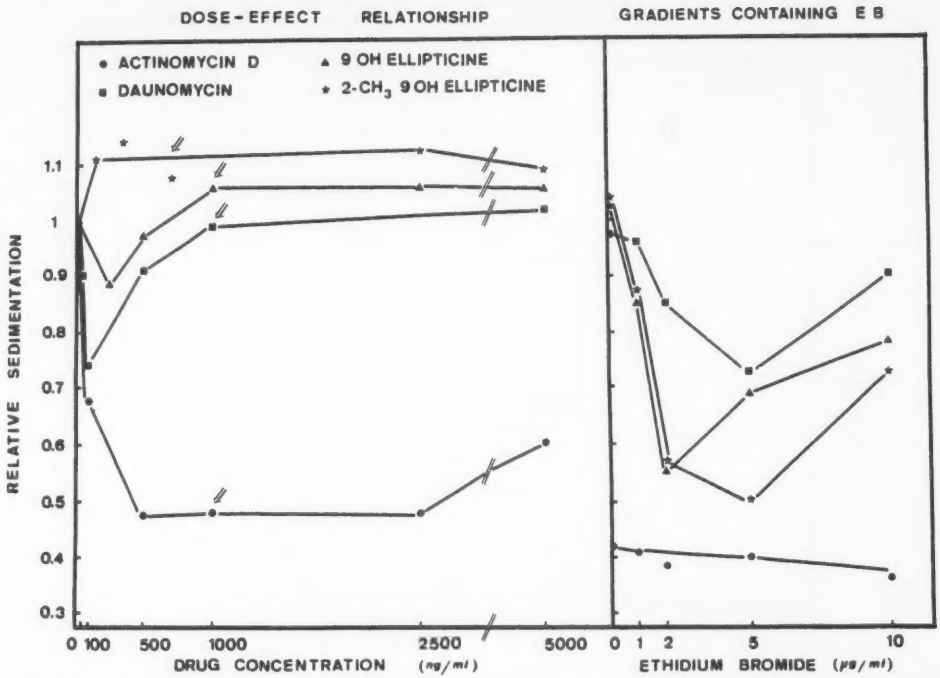


FIGURE 3.—Neutral sucrose sedimentation of DNA extracted from in vitro-grown L1210 cells exposed to 9-hydroxy and 2-methyl-9-hydroxyellipticinium.

Same culture conditions as in figure 2 except that no prelabeling of DNA was performed. Aliquots of resuspended cells were exposed for 1 hr to 9-hydroxyellipticine, 2-methyl-9-hydroxyellipticinium and to actinomycin D and daunomycin at increasing concentrations, selected according to the ID_{50} (drug concentration that reduces by 50% the growth rate of cells exposed for 48 hr). ID_{50} were respectively: 3.9; 13.8; 1.25, and 26 ng/ml).

50 μl of resuspended cells (4×10^5) were lysed during 15 min at 20 °C on linear neutral gradient (15%–30%) in 1.95 M NaCl, 0.01 M Tris (pH 8.0) and 0.001 M EDTA. The lysis solution (150 μl) was made of Triton X-100 0.5% in 1.95 M NaCl, 0.002 M Tris and, 0.05 M EDTA. Centrifugation was run for 35 min at 20 krpm in Spinco SW50.1 rotor at 20 °C. Absorbance of each fraction (UV absorbance monitor Isco) was measured at 254 nm. For one drug concentration (≈) the sedimentation experiments were carried out in sucrose gradients containing 1, 2, 5, and 10 μg/ml of ethidium bromide (right insert).

of pH; (*b*) during cell lysis in the alkaline medium, the release of the majority of the proteins and lipids engaged in nuclear DNA complexes occurs, but the releasing process does not remove the whole set of associated proteins and lipids from DNA (21), so DNA isolated by procedures involving elevated ionic strength and high NaOH concentrations (generally considered as the most efficient ones for purifying DNA) still contains components that might influence the rate of DNA sedimentation in a sucrose gradient. If the content, nature, or mode of binding to DNA of these residual proteins or lipids were modified because of the exposure of cells to ellipticine, the rate of migration of DNA in the gradient might be reduced. Similar differences in the sedimentation pattern of DNA, according to the pH of the sucrose gradient, have been reported for two other antitumor drugs, daunomycin (22,23) and AMSA (24).

The duration of exposure of L1210 cells to ellipticine or to its two hydroxylated derivatives that is required to observe a significant shift of DNA in the alkaline sucrose sedimentation pattern is of the order of a few seconds. After a few minutes of exposure of the cells to relatively high concentrations, ellipticine (60 μg/ml), 9-OH-ellipticine (1 μg/ml) or 2-CH_3-9-OH-ellipticinium (3 μg/ml) do not further modify the DNA sedimentation patterns. The brevity of exposure time of cells to ellipticine, necessary to modify some cellular activities, is a remarkable phenomenon, which has already been reported for ellipticine itself (19) and other antitumor drugs such as daunomycin (22) and AMSA (24).

Regarding the relationship between the drug concen-

tration and the biological effects, the following conclusions have been reached. The concentrations of ellipticine, 9-OH-ellipticine, and 2-CH_3-9-OH ellipticinium that allow the first detectable changes of the alkaline sedimentation patterns of DNA are respectively 0.2 μg/ml, 0.025–0.050 μg/ml, and 0.07 μg/ml after an exposure of 15 minutes of the L1210 cells to the two former drugs and of 60 minutes to the later one. On the other hand, the drug concentrations that bring about the first measurable decrease of the growth rate of the L1210 cells in vitro and of their viability in terms of ability to yield tumors after grafting them back in mice (both parameters are correlated in this system (4)), have been measured. After an exposure of the cells for 15 min, these concentrations are 3.5 μg/ml (ellipticine) and 0.05 μg/ml (9-OH-ellipticine) and, after an exposure of 60 minutes, 0.2 μg/ml (2-CH_3-9-OH-ellipticinium) ((4) and unpublished data).

The following conclusion can be drawn from the comparison of the two sets of data: the modifications of the alkaline gradient pattern of DNA generated by ellipticine and its two hydroxylated derivatives apparently occur at a drug concentration that does not alter some of the most sensitive biological functions of the cells related to their capacity to survive and divide. These biochemical modifications might therefore be equally well considered as an exceedingly sensitive test for monitoring the biological damages suffered by L1210 cells exposed to these drugs or, on the contrary, as events unrelated to the true biochemical reactions that trigger cell damages.

An interaction between the living cells and the drugs is required for the subsequent decreased mobility of DNA in alkaline sucrose gradients. If ellipticine, 9-OH-ellipticine, or 2-CH_3-9-OH-ellipticinium are injected into the lysis medium immediately after the addition of the cells, no change of sedimentation patterns is observed. Evidently such changes do not depend on a chemical reaction arising in the lysis medium or in the gradient. This control was essential because, at alkaline pH, 9-OH-ellipticine and 2-CH_3-9-OH-ellipticinium are auto-oxidized and produce some free radicals (unpublished data). These radicals are very reactive and could have broken DNA during the lysis and sedimentation operations.

INTERACTION OF ELLIPTICINE AND 9-HYDROXYELLIPTICINE WITH PHOSPHOLIPID MONOLAYERS IN VITRO

Figure 1 shows that ellipticine has a net amphipathic character; it tends to become protonated at the N2 position with pK_a values of 6.8. This chemical feature might endow the compound with the ability to interact with membrane components, especially with phospholipids.

As expected, ellipticine dissolved in an aqueous subphase interacts strongly with acidic phospholipids (phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidic acid, and cardiolipin) layered as monomolecular films. The zwitterions phosphatidylcholine and phosphatidylethanolamine, however, are very poor ligands. Ellipticine/acidic lipids interactions bring about large film expansions ($\triangle\pi \sim$ 12 to 22 mM x m^{-1}) and a considerable increase of the surface potential ($\triangle$ ($\triangle$ V) ~ 250 to 300 mV) (fig 4). These interactions are not disturbed by the presence of cations in the subphase at concentration below 10^{-1}M for Na^+ and 10^{-2}M for Ca^{++}. Using the same criteria, no significant drug-lipid interactions could be detected for the dipolar 9-hydroxyellipticine.

These ellipticine-phospholipid interactions occur regardless of the film surface pressure or the nature and physical state of the lipid acyl chains. The molar ratio of the drug/lipid complex is about 1:1 at saturation (fig 5). Phospholipid monolayers are nearly saturated with ellipticine at this equimolar ratio for a drug concentration of 10^{-6}M in the subphase. However, our data show that this interaction cannot be described in terms of an equilibrium between ellipticine molecules bound to the lipid film and those dissolved in the subphase. The interaction leads to a rapid and apparently irreversible binding. For a drug concentration of 10^{-7}M, the interaction is scarcely detectable, presumably because of the slow diffusion rate of the drug in the aqueous phase. Ellipticine also causes a large condensation for both dilauroyl and dipalmitoylphosphatidylglycerol. In the mixed drug/lipid films, both lipids would exist in a very similar and rather condensed state intermediate between the liquid expanded and gel states in which these two lipids can respectively exist in pure water (fig 6). In this respect, ellipticine might have an effect on lipids quite similar to that of cholesterol (25). The binding is stabilized by both electrostatic and hydrophobic forces: the ellipticine molecules can be viewed as inserted between the lipid molecules, the NH^+ group interacting with the phosphate function and the aromatic moiety being in contact with the lipid acyl chains.

These data suggest that the disturbance of the organization and the fluidity of the lipid matrix in membranes might explain some of the effects of ellipticine in biological systems. Such disturbances might either be directly responsible for the loss of some essential cell functions or might alter reactive membrane sites and subsequently expose them to extrinsic agents. These observations also support the hypothesis that some lipids could serve as a reserve binding pool for ellipticine and some of its hydrophobic derivatives after injection in animals, as suggested by pharmacokinetic data (26,28). Finally, drug-membrane interactions might explain the marked differences between the

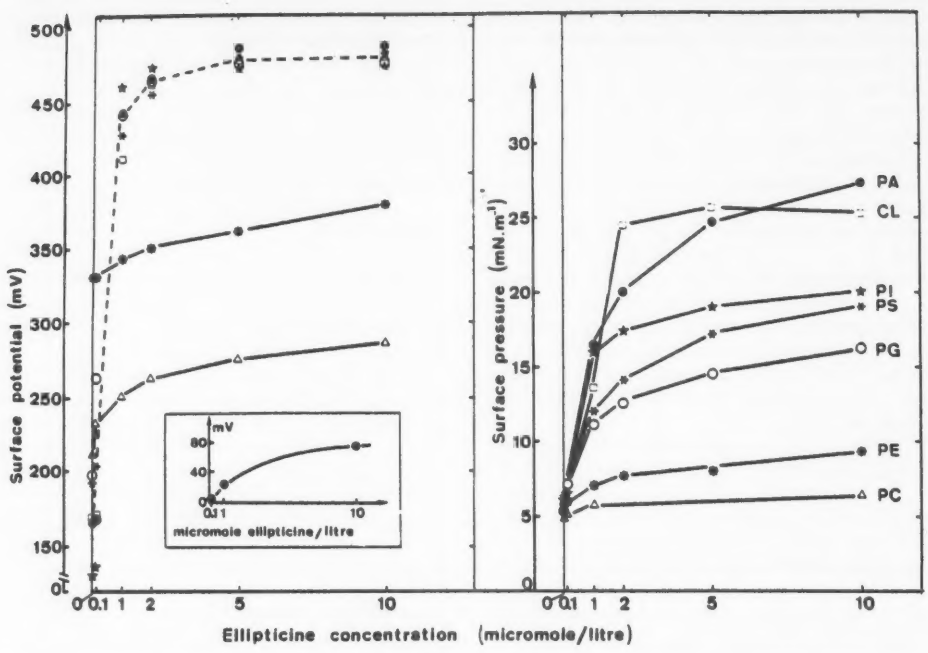


FIGURE 4.—Change of surface pressure and surface potential in monolayer of various phospholipids, as a function of the concentration of ellipticine in the subphase. △ = phosphatidylcholine (PC); * = phosphatidylethanolamine (PE); ○ = phosphatidylglycerol (PG); ⁎ = phosphatidylserine (PS), ★ = phosphatidylinositol (PI); ■ = cardiolipin (CL); ● = phosphatidic acid (PA). The surface pressure was measured by means of a floating barrier. Subphase: 10^{-2}M NaCl at pH 6.0. For experimental details *see* (8,9).

cytotoxicities (4), the antitumor properties (29), and the pharmacokinetic behavior of ellipticine and 9-OH-ellipticine (26,28).

OXIDATION OF 9-HYDROXY DERIVATIVES OF ELLIPTICINE BY PEROXIDASE-H_2O_2 SYSTEM

Ellipticine is metabolized in animal species into hydroxylated derivatives among which 9-hydroxyellipticine is the major product (30–32). Such a process might be of great pharmacological significance since it has been shown that the presence of a hydroxyl group in the 9 positionof both ellipticine and 2-CH_3-ellipticinium leads to a marked increase of cytotoxicity (by a factor of 66 and 31 respectively) (4) and to an improvement of the antitumor efficiency in the case of L1210 mouse leukemia (12). Among several hypotheses that could explain this later observation, we are currently investigating the possibility that the pharmacological properties of the 9-hydroxylated derivatives in this series might be due to their ability to generate in situ highly reactive intermediates by means of a bio-oxidative process. Similar mechanisms have been proposed for some other antitumor drugs (33) such as mitomycin C (33), streptonigrine (34), and anthracyclines and related compounds (35).

As a result of these investigations, we have established that 9-hydroxyellipticine and 9-hydroxyellipticinium are oxidized in presence of peroxidases, enzymes that catalyze the oxidation of various organic compounds with suitable electron releasing groups in the presence of H_2O_2 as an electron acceptor. These enzymes were selected for this study because phenols usually act as substrates for peroxidases, resulting in the generation of reactive metabolites (36). As expected, red colored compounds are produced when peroxidase is allowed to act on 9-hydroxyellipticine and 2-methyl-9-hydroxyellipticinium in the presence of H_2O_2 (fig 7A).

In the case of 9-hydroxyellipticine, the corresponding quinone imine structure (9-oxoellipticine) has been identified by NMR and mass spectrometry. Immedi-

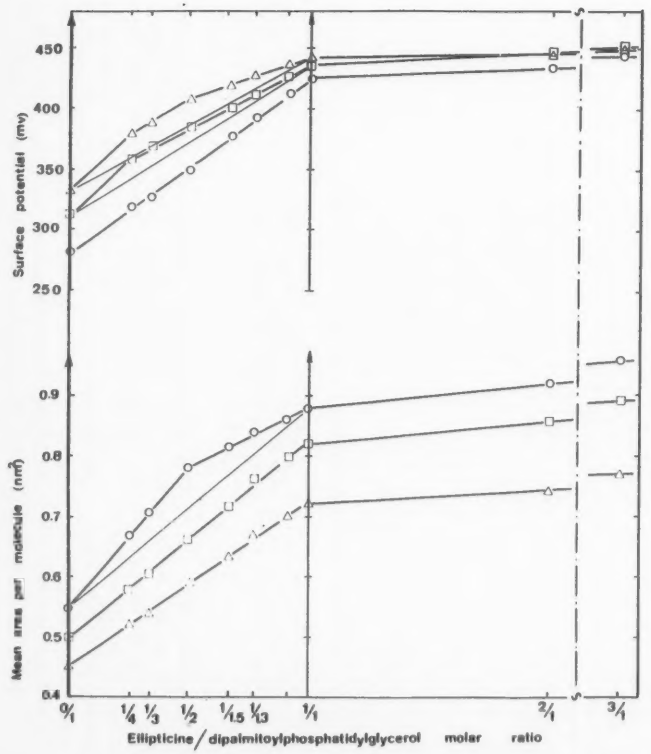


FIGURE 5.—Interaction of ellipticine with dipalmitoylphosphatidylglycerol monolayers. Changes in molecular area and surface potential as a function of drug/phospholipid ratio. Spreading of the phospholipids progressively enriched in drug on 0.001 M NaCl at pH 5.6. Ellipticine was not detected in the subphase and did not form a film. Molecular areas calculated at constant surface pressure of ¶ = 10 mN m^{-1}(¶), - = 15 mN m^{-1} (○) and ¶ = 25 mN m^{-1} (△) from compression isotherms (*see* fig 6). Values refer to films of phospholipids compressed at the same ¶ values. For experimental details, *see* (8,9).

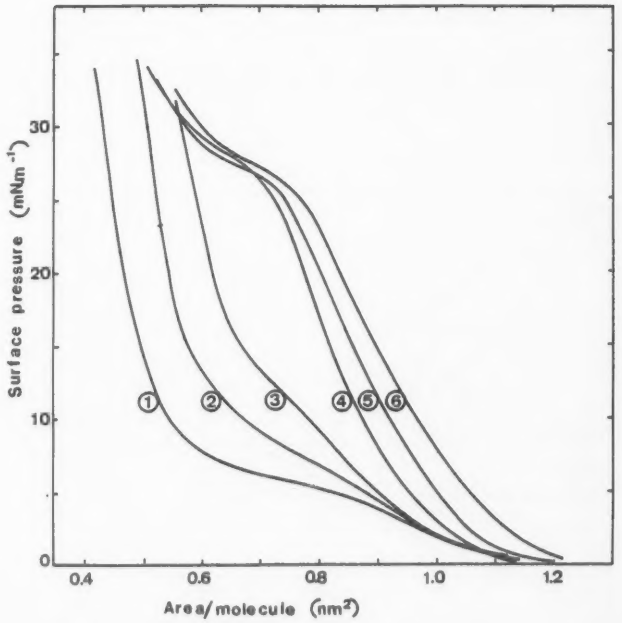


FIGURE 6.—Interaction of ellipticine with dipalmitoylphosphatidylglycerol monolayers. Compression isotherms. Mixtures of ellipticine and phospholipid were spread on 0.01 NaCl pH 5.6 subphase at molar ratio of 1) 0/1, 2) 1/4, 3) 1/2, 4) 1/1, 5) 2/1 and 6) 3/1. Curves were calibrated as if the lipid were alone. For experimental details *see* (8,9).

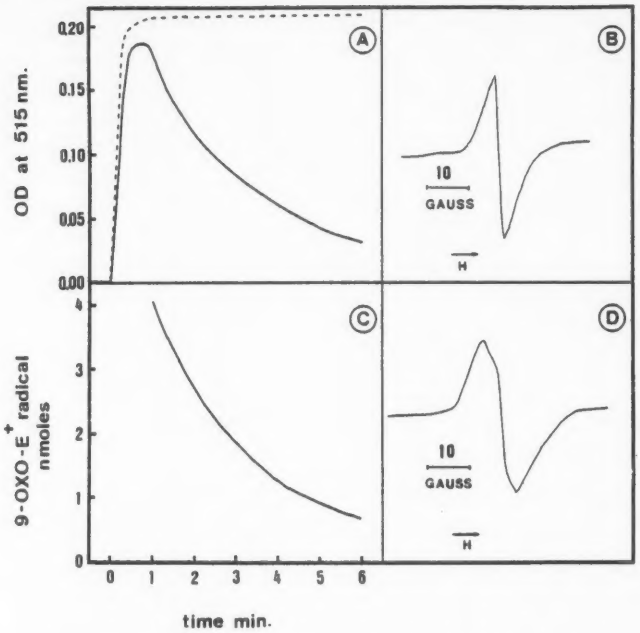


FIGURE 7.—Oxidation of 9-hydroxyellipticine and 9-hydroxyellipticinium catalyzed by MPO-H_2O_2 system.

A. Kinetics of 9-hydroxyellipticine (- - - -) and 9-hydroxyellipticinium (——) oxidations. Experiments were performed at 25 °C in 0.05 phosphate buffer (pH 7.40) in the presence of 10^{-4}M H_2O_2 and 25 × 10^{-6}M 9-hydroxyellipticine or 2-methyl-9-hydroxyellipticinium. At 0 min 2 × 10^{-6}M MPO was added.

B. EPR spectrum of 9-hydroxyellipticine or 9-hydroxyellipticinium radical observed in the mixture described in A when quick frozen in liquid nitrogen 10 sec after the addition of MPO. The spectra were recorded at 110 °K with a microwave frequency of 9750 GHz, 1 Gauss modulation, and 250 mW microwave power. Gain was 8 × 10^4. Both 9-hydroxyellipticine and 9-hydroxyellipticinium radicals were detected at $g = 2.0042 \pm 0.0002$.

C. Kinetics of 9-hydroxyellipticinium radical degradation at room temperature in a mixture as described in A. The decay of EPR signal was recorded 1 min after the addition of MPO using a microwave frequency of 9753 GHz, 1 Gauss modulation, and 250 mW microwave power. The radical concentrations were determined by comparison with a standard solution of spin-ladel material [3-(2-iodacetamido)-2,2,5,5-tetramethyl-l-pyrolidinyloxyl]. The decay of the radical showed first-order kinetics with a rate constant of 0.36 min^{-1}. In similar experimental conditions no signal was observed with 9-hydroxyellipticine.

D. EPR spectrum of 9-hydroxyellipticinium radical at room temperature in the conditions described in C. The spectrum was recorded 5 min after the addition of MPO in the mixture previously sparged with argon. The signal was detected at $g = 2.0046 \pm 0.002$.

ately after the onset of the enzymatic oxidation, free radicals can be detected by EPR spectroscopy at low temperature (fig 7B). The g-factor of 2.0042 suggests that a phenoxy free radical is generated. Moreover, the quinone product of 9-OH-ellipticinium is an unstable compound that gives rise to another radical species during the fast reactions to which it is submitted, yielding insoluble dimers and polymers (fig 7C and 7D). This free radical can be detected at room temperature, its g-factor is 2.0046. A polarographic study has shown that the redox potential of the couple 9-OH/90 is Eo' = + 0.50 v. This value indicates that the oxidized products of 9-hydroxyellipticine should be strong oxidizing species. This property might explain why 9-OH-ellipticine promotes the oxidation of NADH at neutral pH in the presence of a peroxidase-H_2O_2 system (fig 8). Dur-

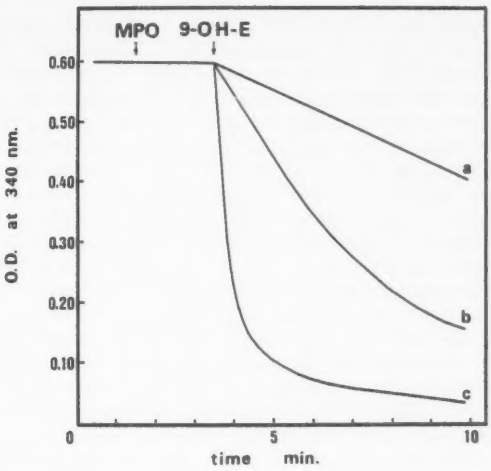


FIGURE 8—Effect of 9-hydroxyellipticine on NADH oxidation catalyzed by MPO-H_2O_2 system at neutral pH. Reactions were carried out at 25 °C in 0.05 M phosphate buffer (pH 7.40) containing 10^{-4}M H_2O_2 and 10^{-4}M NADH. Where indicated, 10^{-5}M 9-Hydroxyellipticine was further added in all cases. In these experimental conditions the apparent K_m of MPO-9-hydroxyellipticine system for NADH has been estimated to be 18 $\times 10^{-6}$M.

ing this oxidation, no modification of structure and no variation of concentration of 9-OH-ellipticine were recorded; therefore, this drug might be considered as a catalyst of NADH oxidation in this system. This observation might explain why only an unchanged product (except for glucuroconjugation) can be isolated after administration of 9-OH-ellipticine or 2-CH_3-9-hydroxyellipticinium to animals or humans. Although the bio-oxidation of 9-hydroxyellipticine and 9-hydroxyellipticinium has not been demonstrated in a biological system, it provides a model that might be lead to understanding of the greater cytotoxicity and anti-tumor properties for 9-hydroxylated derivatives compared to their ellipticine counterparts.

This model has some support from the observation that 7-hydroxyellipticine, which is 360 times less cytotoxic than 9-hydroxyelipticine (4), is not a substrate for peroxidase and therefore does not seem to be able to produce a free radical.

INTERACTION OF ELLIPTICINE AND 9-HYDROXY DERIVATIVES WITH CYTOCHROME P450 MIXED MONO-OXYGENASES

Due to its physicochemical characteristics (hydrophobicity of the polyaromatic structure and the pyridine nucleus), ellipticine is expected to a be a good ligand for cytochrome P450, the terminal catalytic component of microsomal mono-oxygenase complexes. This assumption has been verified by spectral interaction studies of these drugs with liver microsomes (11). Ellipticine binds strongly to oxidized and reduced forms of cytochrome P450 producing typical difference spectra with peaks at 428 nm for the oxidized form and 445 nm for the reduced form. These type II difference spectra are similar to those produced by compounds containing one pair of electrons on the nitrogen atom and probably correspond to the binding of the ellipticine pyridine nitrogen to the sixth coordination site of cytochrome P450 iron. Ellipticine has a high affinity for microsomal cytochrome P450. For instance, its apparent spectral dissociation constant, K_s, is 4.3×10^{-7}M for the oxidized form of noninduced rat liver microsomes. This affinity is slighty lower for Aroclor-induced microsomes, with a K_s of 7.5×10^{-7}M. The same interaction properties have been also demonstrated with partially purified rabbit lung cytochrome P450 (37) as well as human liver microsomes (unpublished results).

9-Hydroxyellipticine also binds to cytochrome P450. Its apparent spectral dissociation constants are respectively 6.5×10^{-7}M and 5.2×10^{-7}M for noninduced and benzo(a)pyrene-induced rat liver oxidized microsomes. It is only 3.7×10^{-5}M for reduced phenobarbital-induced microsomes.

On the other hand, 2-methyl-9-hydroxyellipticinium is unable to elicit an interaction spectrum with microsome suspensions, and no evidence of an interaction of this charged mulecule with cytochrome P450 has been obtained.

The strong binding of ellipticine and 9-hydroxyellipticine to reduced heme iron of cytochrome P450 probably prevents the fixation and activation of molecular oxygen. According to this hypothesis, these drugs are expected to inhibit a large number of cytochrome P450-dependent mono-oxygenases. We have shown (38) that aminopyrine-N-demethylase, aniline hydroxylase, and benzo(a)pyrene hydroxylase activities were strongly inhibited by 9-hydroxyellipticine with I_{50} values from 0.3 to 4.5 mM, depending on the substrate and the type of induction of microsomes. By comparison with standard inhibitors of the forms of cytochrome P450 induced either by phenobarbital (metyrapone) or by polycyclic hydrocarbons (7,8-benzoflavone), it appears that 9-hydroxyellipticine is about ten times more potent an inhibitor in terms of the amount of drug needed to obtain the same effect. So, some ellipticine derivatives represent a class of very powerful inhibitors of the whole set of cytochrome P450-dependent activities of rat liver microsomes.

These properties have two pharmacological implications:

1. Several studies carried out either in vivo on rats (30,32) or in vitro on rat liver microsomes (31) demonstrate that ellipticine is oxidized to various metabolites that are mostly eliminated in the bile. The major metabolite is 9-hydroxyellipticine. A minor metabolite is 7-hydroxyellipticine. Since these two compounds display large differences of cytotoxicities, as shown above, it is noteworthy that the relative yield of these two derivatives after injection to animals can be modulated by inducers of the various forms of cytochrome P450. For instance, the pretreatment of rats by Aroclor or 3-methylcholanthrene, which induces the synthesis of cytochromes P448 and "P_1 450" (39), leads to a larger proportion of 7-hydroxyellipticine than in control rats. In contrast, pretreatment by phenobarbital strongly reduces this proportion. Thanks to the biochemical background provided by the drug microsome interaction studies whose results were briefly summarized above, we have been able to reconstitute the metabolic machinery responsible for the biotransformation of ellipticine: in vitro, in the presence of rat liver microsomes, part of the ellipticine bound to cytochrome P450 is transformed into free 9-hydroxyellipticine (major product) and free 7-hydroxyellipticine (minor product) in the presence of NADPH. Collectively, these experiments demonstrate that ellipticine has an high affinity for the cytochrome P450 mixed oxygenases and can be acted upon by these enzymes. However, its transformation is self-inhibited at high concentration because ellipticine is also an inhibitor of these enzymes (fig 9). This drug concentration-dependent action of ellipticine might have significant pharmacologic implications. In similar experimental conditions, 9-hydroxyellipticine does not yield any detectable metabolite. However, this drug very probably undergoes some NADPH-dependent transformation since about 10% of it becomes nonextractable by the usual organic solvents after being put in contact with the microsomes. This time-dependant phenomenon is prevented by α-tocopherol, a scavenger of free radicals, and by metyrapone. Considering the data that were presented above (see section on the peroxidase-H_2O_2system), it is therefore likely that some oxidation-reduction process of 9-hydroxyellipticine occurs in presence of NADPH-microsomes.

2. The ability of ellipticine and some of its derivatives to block the cytochrome P450-mediated oxidative metabolism in microsomes makes these compounds able to inhibit the microsome-mediated mutagenicity of a great number of compounds belonging to various chemical classes (aromatic amines, polycyclic aromatic hydrocarbons, fungal toxins, and azo compounds (11,40)). This property, which was established using the Ames test, might have been anticipated since a wide variety of these environmental chemicals are mutagens only after a metabolic activation mediated by the same microsomal cytochrome P450-dependent mono-oxygenases, which are in fact strongly inhibited by ellipticine and several derivatives. Parallel studies have also shown that ellipticine is able to inhibit the in vitro covalent binding to DNA of reactive metabolites of benzo(a)pyrene after activation of this hydrocarbon by rat liver microsomes (11). The I_{50} of such binding lies around 5 μM. 7,8-Benzoflavone, a well known inhibitor of cytochrome P448, displays less inhibitory capacity than ellipticine. 9-Hydroxyellipticine has the same properties at even smaller doses. The amount of this drug that decreases the mutagenic activity of the various compounds previously mentioned in the Ames test is lower than the amount of the mutagen itself used in the assay. Due to the good correlation between the mutagenic and carcinogenic properties of these chemicals, it is likely that an antimutagenic drug might also be able to exert an anticarcinogenic effect. This assumption has been effectively verified: the rate of tumor formation in male NMRI Swiss mice caused by repeated skin applications of 7,12-dimethylbenz(a)-anthracene is reduced by the simultaneous application of 9-OH-ellipticine (41).

CONCLUSION

Ellipticine displays many biochemical properties that might have pharmacologic significance. It binds to DNA in vitro and modifies the DNA or the DNA complexes in treated L1210 cells. Nevertheless, it does not break DNA in these conditions. It displays a rather high affinity for phospholipid monolayers and interacts strongly with the microsomal cytochrome P450 system. Other properties of biochemical interest are reported in the literature. Ellipticine, like other DNA intercalating drugs, affects RNA metabolism; the synthesis of ribosomal precursors may be inhibited more strongly than the synthesis of mRNA precursors (42). It also inhibits RNA methylases in isolated nuclei (43); unexpectedly, 9-hydroxyellipticine and 2-methyl-9-hydroxyellipticinium stimulate methylation of histones by S-adenosylmethionine (44). It also interferes with DNA binding to the uterine estradiol-receptor complex (45). Such diversity of action raises unsolved questions about the actual target(s) of this drug, especially since the concentratons of ellipticine that elicit many of these effects lie in similar range (about 10^{-6}M).

Many derivatives of ellipticine have been prepared. Tests for their biochemical and/or antitumor properties (46) have shown that these compounds might reveal major differences in biochemical reactivity compared to that of ellipticine itself, despite their close structural similarity to ellipticine. For example, 9-bromoellipticine is the only derivative in this group of substances that is able to induce breaks on DNA in cells (unpublished data); the hydroxylation in position 9 of ellipti-

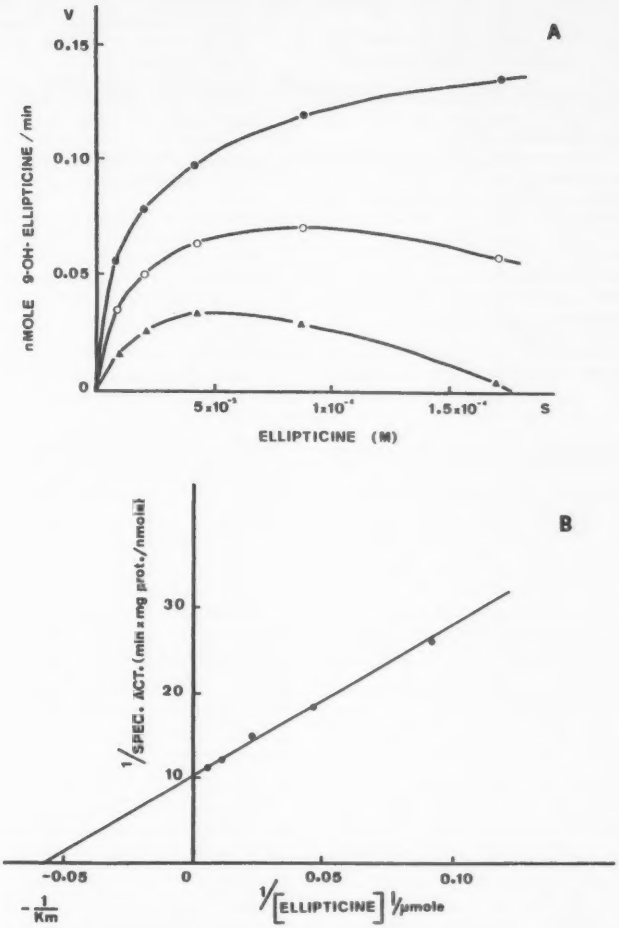


FIGURE 9.—Kinetic data of ellipticine hydroxylation by microsomes of control rat.

A. Effects of microsomal protein and substrate concentrations. The amount of 9-hydroxyellipticine formed in 1 min is plotted against substrate concentration. The protein concentrations were 1.5 mg (●), 1 mg (○), and 0.5 mg (△) per ml respectively.

B. Determination of K_m according to Lineweaver-Burke plot. The protein concentration was 1.5 mg/ml.

cine suppresses its capacity to bind to acidic phospholipids and yield a structure prone to oxidation-reduction processes; the quaternarization of pyridine nitrogen of ellipticine prevents any interaction with the cytochrome P450 microsomal mixed oxygenase system. The only guideline that emerges from this wealth of data is that a relatively high affinity of ellipticine derivatives for DNA is a prerequisite for the occurrence of cytotoxic and antitumor properties (11). However, no clear structure-activity relationship can yet be proposed, despite the apparent simplicity of this pyridocarbazole structure compared to that of other antitumor drugs of natural origin.

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Biochemical and Pharmacologic Properties of Nitrosoureas[1]

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This review describes current research to improve our understanding of nitrosourea antitumor activity as well as attempts to decrease selectively the myelosuppressive properties of this class of alkylating agents.

In 1959, 1-methyl-1-nitroso-3-nitroguanidine (MNNG) was reported to possess measurable and reproducible antitumor activity against the murine ascitic leukemia L1210 (1). This observation did not result in clinical trials of MNNG, but it encouraged an intensive evaluation of N-nitroso compounds for antitumor activity. It was demonstrated that the nitroso group of MNNG was required for activity and that substitution of a chloroethyl group for the methyl group increased its cytotoxicity. Ultimately this led to the synthesis of 1-(2-chloroethyl)-nitrosourea (CNU), which had curative activity in the L1210 system (2).

Another approach to this problem was employed by Montgomery and colleagues who synthesized compounds with structural similarities to MNNG and which could potentially release diazomethane. 1-Methyl-1-nitrosourea (MNU), the first nitrosourea studied, was found to be more active against ip implanted L1210 than MNNG and also had activity against intracranially implanted L1210, which was related to its lipid solubility and resultant ability to cross the blood-brain barrier (3). This work led to the synthesis of more than 200 nitrosoureas and ultimately to the development and clinical use of a series of chloroethyl nitrosoureas including 1,3-bis(2-chloroethyl)-1-nitrosoureau (BCNU) (4), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and eventually methyl-CCNU. These drugs proved to be active in a wide range of human tumors (5). However, they all produce delayed and cumulative bone marrow toxicity, which has seriously limited their clinical application.

Streptozotocin formed by attaching MNU to the C-2 position of glucose and found as a naturally occurring fermentation product of *Streptomyces achromogenes*, is active in L1210 and exhibits far less myelosuppression than MNU (6). This observation led to the synthesis of a chloroethyl derivative, 2-[-3-(2-chloroethyl) -3-nitrosoureido]-D-glucopyranose (chlorozotocin) (7), which also exhibits reduced myelotoxicity at maximally effective doses in the L1210 system (8).

METABOLISM OF NITROSOUREAS

Nitrosoureas spontaneously decompose under physiological conditions (9,10), with chemical half-lives in phosphate buffered saline (pH 7.4) ranging from 5 minutes for CNU to 2 hours for CCNU (11). During the degradation process, a series of alkylating moieties are formed, of which the highly reactive alkyldiazo-hydroxide and carbonium ion are considered the most important. Organic isocyanates that also are generated carbamoylate intracellular proteins (12,13). In addition to this decomposition, nitrosoureas are metabolized by the hepatic mixed function oxidase system to more polar hydroxylated products, which retain both the cyclohexyl ring structure and the cytotoxic nitrosoureido moiety (14,15). Studies of CCNU metabolites, following a 1-hour slow intravenous infusion in man, have shown that 75% of plasma drug concentration was in the form of hydroxylated products (16). Of these, two-thirds appeared as trans-4-hydroxy CCNU and one-third as the cis-4-isomer.

Because the rate of metabolic hydroxylation of CCNU exceeds the rate of chemical dissociation, the hydroxylated products mediate both the therapeutic and toxic activities. Wheeler (17) has shown that these hydroxylated CCNU derivatives are, in general, more water soluble, and have higher in vitro alkylating activities, and several have low carbamoylating activities. In addition to hydroxylation of ring structures, Hill et al have shown that both CCNU and MNU are denitrosated by liver microsomes (18). As additional supportive evidence of metabolism, all clinically-used nitrosoureas, with the exception of ACNU, exhibit a greater mutagenic activity (12%–40%) after incubation with liver microsomal fraction S-9 (19). No direct relationship between in vitro alkylating activity and mutagenic potential of the nitrosoureas has been found. However, the products after S-9 metabolism have mu-

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tagenic activity equivalent to that of activated 3-methylcholanthrene (19). This may be of importance in regard to potential carcinogenic activity in man.

ALKYLATION AND CARBAMOYLATION

The degradation products formed during chemical decomposition result in moieties that alkylate and carbamoylate cellular macromolecules. In general, amino, carboxyl, sulfydryl, and phosphate groups are targets for alkylation. Carbamoylation occurs at sites of "active" hydrogen groups, such as the ϵ-amino groups of lysine or arginine. Alkylation is widely accepted as the principal mechanism of antitumor activity, but our studies have failed, with a wide range of nitrosoureas, to show a linear relationship between in vitro alkylating activity and the chloroethyl antitumor activity for the murine L1210 leukemia (20,21). Studies with water-soluble analogues have suggested a parabolic relationship with an optimal relative alkylating activity of 60% of that of chlorozotocin (20,21). In addition, there was no correlation between carbamoylating activity and antitumor activity (22), granulocyte suppression (20), or lethal toxicity (21). Both chlorozotocin and streptozotocin possess negligible carbamoylating activity but retain antitumor activity. The implication from this is that the contribution of carbamoylation to cytotoxicity is minor at best.

Kohn has proposed that the single alkylation function of the chloroethyl nitrosoureas can cross-link DNA as determined by inhibition of alkali-induced strand separation (23). This cross-linking is the result of a chloroethylation of a nucleophilic site on the first strand, followed by a displacement of Cl^- by a nucleophilic site either on the same or on an opposing DNA strand, forming an ethyl bridge. There also can be linkage between DNA and nuclear proteins, as demonstrated by binding to specific proteins. This process of DNA-DNA and DNA-protein cross-linking may contribute to the overall mechanism of toxicity, along with a combination of macromolecular alkylation and carbamoylation.

EFFECT OF CARBAMOYLATION ON DNA REPAIR

It has been shown that nitrosoureas with high carbamoylating activity delay the repair of x-ray and uv-irradiation damage to DNA. Kann et al have demonstrated that repair of x-irradiation damage of L1210 DNA can be inhibited in vitro by organic isocynates (24). The authors attributed previous equivocal results to the phenomenon of DNA-protein cross-linking, which greatly decreased the measurable DNA repair as determined by the alkali elution method. In cell lysates pretreated with proteinase K, prior to alkali elution, the DNA segments that were attached to proteins, but not repaired, eluted more rapidly than the cross-linked DNA-protein complexes. Not only was the concept of nitrosourea repair inhibition confirmed, it was also demonstrated that drugs with the highest carbamoylating activity were active at the same molar concentrations required by isocyanates (25).

The mechanism by which nitrosoureas inhibit repair synthesis following x-irradiation damage has recently been described. Fornace et al (26) showed that 2-chloroethyl isocyanate, a breakdown product of BCNU, interfered with the polynucleotide ligase step of DNA excision repair. In normal human fibroblasts exposed to uv-irradiation, the initial enzyme in repair, uv endonuclease, was not inhibited. Repair polymerase and repair replication activities were also shown to be unaffected. By exclusion, the final enzymatic step of nucleotide ligase was implicated as the site of inhibition by chloroethyl isocyanates in this system. This is consistent with the inactivation of other cellular enzyme systems by carbamoylating agents (27).

In contrast to x-irradiation damage to DNA, the significance of carbamoylating activity on the repair of alkylation damage by the same drugs has been questioned. Heal et al (28) showed clear evidence of inhibition of DNA repair of nitrosourea DNA alkylation with 3-β-D-glucopyranosyl-1-methyl-1-nitrosourea (GMNU), an agent with a relatively high carbamoylating activity. However, there was no increase in in vitro L1210 cytotoxicity or in vivo L1210 antitumor activity compared to streptozotocin, a nitrosourea with an equivalent alkylating activity and little carbamoylating activity. Thus the primary antitumor and cytotoxic action of the nitrosoureas is alkylation. DNA repair inhibition by carbamoylation, although probably significant for irradiation injury, does not appear to impart an important intrinsic cytotoxic property.

PYRIDINE INTERACTION

The metabolism of nicotinamide has been shown to be important in both the antitumor activity and cytotoxicity of several chemotherapeutic agents. Early work with cytotoxic agents (29–31) in rodent tumor systems identified either an activity-dependent depression of NAD concentration or the formation of false NAD analogs as the principal mechanism of the cytotoxic action. In all systems studied, there was a reversal of the antitumor activity with the concomitant administration of nicotinamide.

It has been demonstrated that all nitrosoureas and nitrosamines possess an R-N(NO)-CH_3 or -CH_2CH_3 end group that can markedly depress pyridine nucleotide concentrations in many normal tissues, as well as the L1210 leukemia. The addition of a halide, such as Cl^- in the case of BCNU and CCNU, removes this property (32).

The diabetogenicity of streptozotocin showed a direct dose-related correlation with reductions in islet NAD, which could be prevented for the L1210 leukemia in vivo with nicotinamide, without loss of antitumor activity. Later studies showed that this nicotinamide-streptozotocin combination caused pancreatic islet cell adenomas in rats. This is likely due to DNA methylation of beta cell DNA by MNU, the cytotoxic moiety of the streptozotocin molecule, while nicotinamide prevented the acute reduction of pyridine nucleotides that otherwise would have resulted in islet destruction. The chloroethyl nitrosoureas had no effect on intracellular NAD concentrations (32,33). The observed reduction of NAD after methyl nitrosourea exposure has proven to be of interest when correlated with studies of the nuclear nonhistone protein, poly-ADP-ribose polymerase. This enzyme uses NAD as a substrate rather than as a cofactor and consumes considerable intracellular NAD for the modification of various nuclear proteins. The data suggest that the activated enzyme is in part responsible for the profound decrease in cellular NAD.

Treatment of HeLa cells with millimolar concentrations of MNU (34) and streptozotocin (35,36) resulted in a marked activation of the enzyme. Further investigations with the chloroethyl nitrosourea, CCNU, showed a slight inhibitory effect on the enzyme activity at concentrations up to 4 mM (33). However, no relationship between the in vitro alkylation and carbamoylation potential of nitrosoureas and stimulation of poly-ADP-ribose polymerase activity has been shown.

INTERRELATIONSHIP OF NITROSOUREAS WITH CHROMATIN

Chromatin is composed of nucleosomes, which are double tetrameric cores of histones H2a, H2b, H3 and H4 (37) in association with ~ 140 base pairs of DNA. The nucleosomes are separated by linker regions consisting of up to 60 base pairs of DNA with histone H1.

Limit digests of specific regions of chromatin by micrococcal nuclease and DNase I have provided further data on the relative accessibility of chromatin substructures for alkylation: micrococcal nuclease results in preferential digestion of internucleosomal DNA with the production of nucleosomal subunits; in contrast, treatment of chromatin with DNase I initially results in the digestion of nucleosomal core DNA and concomitantly in a preferential cleavage of transcriptional regions of chromatin (38–40).

Nuclei were extracted from HeLa cells treated for 2 hours with 0.3 mM (^{14}C-chloroethyl)-chlorozotocin or (^{14}C-chloroethyl)-CCNU and were subjected to limit digestion with either micrococcal nuclease or DNase I (41). At early time points of digestion (5 min), micrococcal nuclease digested approximately 25% of the total nuclear chromatin from either chlorozotocin- or CCNU-treated cells, with release of less than 10% of total covalently bound ^{14}C-alkyl groups. This suggested that the major alkylation had taken place in a chromatin fraction that was not susceptible to micrococcal nuclease digestion, ie, DNA associated with the nucleosomal core particle. This was confirmed using DNase I, which after 5 minutes of digestion released greater than 35% of the alkylated chromatin as compared to 20% of total nuclear chromatin. Thus both digestions were consistent with a preferential CCNU and chlorozotocin alkylation of L1210 and HeLa cell DNA in the nucleosome core. In contrast, we have data suggesting that the pattern of chlorozotocin alkylation in murine bone marrow differs from that of HeLa and L1210 cells (42,43). These data also demonstrate a specific binding to the internucleosomal linker regions. Such findings may prove relevant to the repair potential of alkylated regions of chromatin as well as the reduced myelotoxic properties of chlorozotocin.

Methyl-^{14}C-MNU, in contrast to the chloroethyl nitrosoureas, reacts with the linker regions of DNA between the nucleosomes. It is likely that the dissimilar chromatin binding regions of the methyl and chloroethyl nitrosoureas contribute to the differing cytotoxicity and carcinogenic potentials of these two classes of nitrosoureas.

We have also demonstrated a preferential alkylation of extended (probably transcriptional) chromatin subfractions in HeLa cells by chloroethyl nitrosoureas (44–46). The quantitative alkylation of functional chromatin regions may prove important to the cytotoxic properties of these drugs, although there is no evidence at present for differences between tissues such as tumor cells and bone marrow.

Treatment of cells with sodium butyrate produces chromatin structural alterations secondary to nuclear protein modifications, with a resultant increase in transcriptional activity (47,48). Treatment of HeLa cells is associated with a 1.8-fold increase in cellular uptake of CCNU and chlorozotocin into the cytoplasmic fraction, as well as a 2.4-fold increase in the incorporation into the nuclear fraction (41). Studies are now in progress to determine whether this increase resulted from an effect on nuclear membrane permeability or from an increase in the number of available binding sites within the chromatin structure. The latter explanation has gained credence from the recent observation that butyrate causes single-strand breaks in DNA (49). This could permit unwinding of DNA and thereby expose more target sites for drug action. An alternative explanation may be that the DNA is more available for alkylation because of the increase acetylation of the NH_2-terminal arms of histones, particularly H3

and H4, (50,51); there is evidence that such DNA is more readily cleaved by DNase I (52,53).

It has been found that hydrocortisone, like sodium butyrate, can be used to stimulate transcription (44). This is of interest, since corticosteroids are used clinically in combination with alkylating agents in the treatment of many human malignancies (54). DNase II digests chromatin to produce small fragments with minimal disruption of nucleoproteins. The amount of digested DNA that is Mg^{++}-soluble has been shown to vary quantitatively among tissues, but correlates with the transcriptional activity of the cell under consideration. By treating HeLa cells with 1 μM hydrocortisone it is possible to increase transcription about sixfold. Whole chromatin alkylation by 0.6 mM chlorozotocin or CCNU does not differ significantly from the control values of 132 pmol/A_{260} and 106 pmol/A_{260} respectively. In contrast, at the same concentration of hydrocortisone, alkylation of the DNase II-sensitive, Mg^{++}-soluble chromatin subfraction was significantly increased; for chlorozotocin from 41 to 85 pmol/A_{260} (44). These data provide further evidence for the theory that transcriptional chromatin acts as a preferential target for drug interaction.

Hydrocortisone-induced alterations of chromatin were considered indirectly by studying sensitivity of chromatin from control and hydrocortisone-treated cells to micrococcal nuclease digestion. Cells were preincubated with 0.1, 1.0 and 2.0 μM hydrocortisone, nuclei were extracted, and 10%–30% sucrose density gradient sedimentation of chromatin was performed. The A_{260} profile showed peaks representing small molecular weight material (< 1N), nucleosomal monomers (1N), dimers (2N), and hexamers (6N). These molecular weight patterns were confirmed by polyacrylamide gel electrophoresis. Hormone-chromatin interaction appears to have altered the cleavage sites for micrococcal nuclease, since a hormone concentration-dependent shoulder was produced in the region of 21-S (>2N) (55). Since the structure of H1 is critical to hormone binding to chromatin, the observed shoulder may represent the dimer plus hydrocortisone-receptor complex, which could sediment in this range.

Other data (56) suggest that nucleotide sequence and complexity influence the hydrophobic interaction of the hydrocortisone-receptor complex with DNA. For example, Cake et al (57) proposed that an electrostatic interaction between the hydrocortisone-receptor positive charges and DNA phosphate groups occurred, which was further stabilized by the positive charges of receptor surface lysine residues. By stimulating transcriptional activity with a short-chain fatty acid or a steroid there is an increase in the available "target sites" resulting in increased alkylation or carbamoylation. Thus these manipulations are important for gathering information on the mechanism of nitrosourea action and for increasing the effectiveness of this class of compounds.

The alkylation of nuclear RNA was determined by studies using the enzyme T2 RNase, a nuclease that splits RNa into acid-soluble 3′ nucleotides by nonspecific attack on phosphodiester bonds; ^{3}H-uridine prelabeling showed that 90% of the HeLa cell nuclear RNA was solubilized by the nuclease (44). Covalent binding to nuclear RNA was found to contribute only 10%–20% of total nuclear alkylation, whereas 50% of total cellular alkylation is in RNA (44).

Cytoplasmic protein alkylation comprises 40% of total cellular alkylation (58). At the nuclear level, alkylation of histone and nonhistone proteins by chlorozotocin and CCNU is very low (CCNU, 1.6±0.4 pmol/A_{230}, CLZ, 0.6±0.2 pmol/A_{260}) (41). At 2.2 mM, MNU alkylates H2a and H1, as well as numerous nonhistone proteins (59). Lower concentrations of MNU and CCNU showed carbamoylation of nuclear proteins. MNU carbamoylation predominated in H2b and H1 with lower levels of H4 and nonhistone protein modification. The principal sites of CCNU carbamoylation are the histone-like high-mobility group proteins M1 and M4 and the nonhistone proteins, whereas histone H1 is carbamoylated to a lesser extent.

SUMMARY

The employment of traditional biochemical and pharmacological concepts, together with the development of original molecular approaches, has created a more comprehensive understanding of the mechanism of action of nitrosoureas. For example, it is clear that all nuclear macromolecules are susceptible to alkylation and/or carbamoylation by nitrosoureas. The relative importance of these respective processes is equivocal, but there is evidence to suggest that the alkylating potential of these drugs is directly responsible for their cytotoxic, mutagenic, or carcinogenic properties. The structural organization of nuclear chromatin, either at the nucleosomal or functional level, is a critical determinant when considering specific macromolecules as potential targets for nitrosourea interaction. Perhaps a more complete understanding of the nuclear architecture of specific cell types will lead to a more precise understanding of nitrosourea selectivity, especially with respect to the nonmyelotoxic properties of chlorozotocin. Such knowledge would also assist in the rational development of novel nitrosourea analogues.

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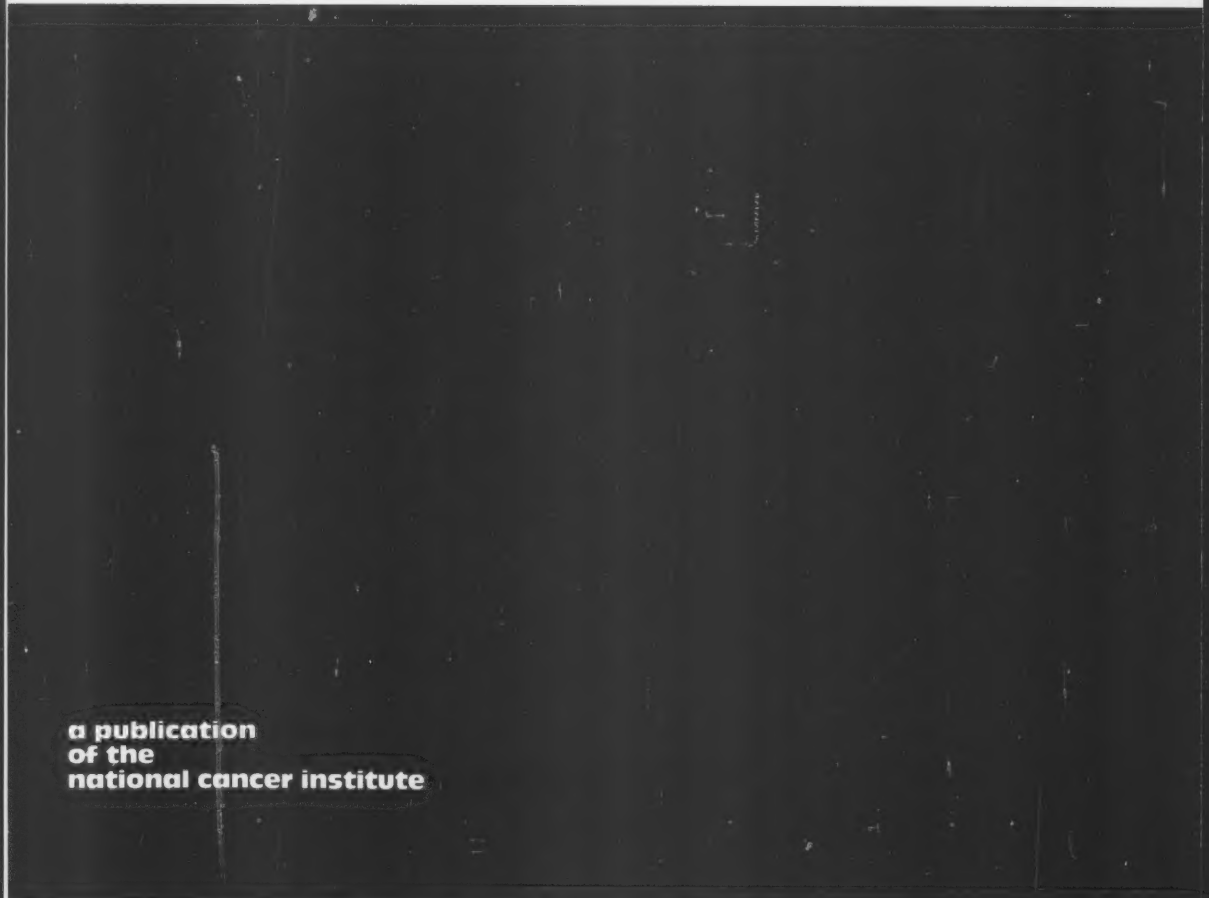

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